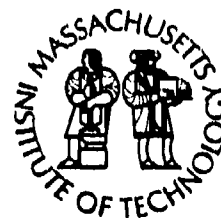


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**Woods Hole Oceanographic Institution  
Massachusetts Institute of Technology**



**Joint Program  
in Oceanography  
and  
Oceanographic Engineering**



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**DOCTORAL DISSERTATION**

**The Life Cycle of the Centric Diatom  
*Thalassiosira weissflogii*:  
Control of Gametogenesis and Cell Size**

by

**E. Virginia Armbrust**

June 1990

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Woods Hole Oceanographic Institution  
Woods Hole, Massachusetts 02543

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Cambridge, Massachusetts 02139

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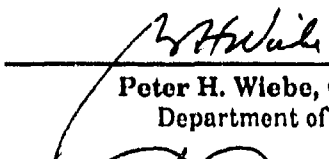
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Peter H. Wiebe, Chairman  
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The Life Cycle of the Centric Diatom *Thalassiosira weissflogii*:  
Control of Gametogenesis and Cell Size

by

ELIZABETH VIRGINIA ARMBRUST

A. B., Stanford University  
(1980)

Submitted in partial fulfillment of the  
requirement for the degree of

DOCTOR OF PHILOSOPHY

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

and the

WOODS HOLE OCEANOGRAPHIC INSTITUTION

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THE LIFE CYCLE OF THE CENTRIC DIATOM *THALASSIOSIRA*  
*WEISSFLOGII*: CONTROL OF GAMETOGENESIS AND CELL SIZE

by

ELIZABETH VIRGINIA ARMBRUST

ABSTRACT

The predominant mode of reproduction in all unicellular algae is via asexual reproduction. In diatoms, the physical constraints of the cell wall, or frustule, during these mitotic divisions generally result in a decrease in the average cell size of a population over successive generations. The most common manner of breaking this trend of diminishing cell size is through sexual reproduction; meiosis replaces mitosis and the resulting male and female gametes fuse to create a zygote or auxospore which then develops outside the confines of the frustule and forms a post-auxospore cell many times larger than either parent. It is traditionally believed that these newly created large cells are unable to undergo sexual reproduction; theoretically, only cells at the lower end of the size spectrum possess this capability. However, most of the studies concerning diatom life cycles are descriptive and in reality very little is known about what determines if and when a cell will undergo gametogenesis and subsequent zygote formation. The motivation behind this research was a desire to understand more about this "decision making" process in centric diatoms.

Using flow cytometric techniques, I showed that the marine centric diatom, *Thalassiosira weissflogii*, can be induced to undergo spermatogenesis by exposing cells maintained at saturating intensities of continuous light to either dim light or darkness. From zero to over ninety percent of a population can differentiate into male gametes depending upon both the induction trigger and the population examined, regardless of cell size. Through the use of populations representing distinct cell cycle distributions, it was shown that responsiveness to an induction trigger is a function of cell cycle stage; cells in early  $G_1$  are not yet committed to complete the mitotic cycle and can be induced to form male gametes, whereas cells further along in their cell cycle are unresponsive to these same cues.

*T. weissflogii* can also undergo sexual reproduction under constant environmental conditions as often as every 120 generations. In the absence of any external induction signals, the ability of a cell to undergo gametogenesis and subsequent auxospore formation is linked with the attainment of an appropriate cell size. However, this permissive size range can vary between isolates and



within a given isolate over time. Moreover, the size of the post-auxospore cells created during these sexual events is also extremely variable; absolute cell size predicts very little about the future conduct of a cell. The unpredictable behavior of these cultures is hypothesized to result from the fact that the genetic composition of a diatom population changes over time. Since diatoms are diploid, each round of sexual reproduction creates genetic diversity, thus enabling the characteristics of a population to undergo frequent transformations.

Thesis Supervisor: Prof. Sallie W. Chisholm  
Professor, M.I.T

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The experiments are completed, the writing is all done (more or less) and the defense presentation is over. It's finally my turn to write an acknowledgment, the one part of a thesis that everyone reads first - hmmm, let's see ... who all helped this person finish? It's my chance to be just as sappy as I want, to thank as many people as I want, in whatever way I want. So with that as an intro, let's see who all helped Ginger finish.

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## Preface

Diatoms are one of the most abundant eukaryotic microorganisms found in the aquatic environment. Due to their photosynthetic capabilities, these ubiquitous organisms are substantial contributors to global primary productivity. And yet, despite their obvious ecological importance, relatively little is known about the details of the physiology of these cells. In fact, studies of the life cycle of diatoms have not progressed beyond the descriptive stage (Chapter 1); very little is understood about what determines if and when a cell will cease asexual reproduction and undergo gametogenesis and subsequent zygote formation. The goal of my thesis was to examine in more detail this "decision making" process.

The marine centric diatom, *Thalassiosira weissflogii*, was used as the model organism for this research due to a serendipitous observation by Vaulot and Chisholm (1987). They found that a portion of a *T. weissflogii* population was induced to undergo spermatogenesis in response to a change in light conditions. What fascinated me about this result was that a majority of the population apparently ignored the induction signal and continued dividing asexually. What differentiated these non-inducible cells from the inducible ones? To address this question, the responsiveness of a cell to an environmental induction trigger was examined as a function of both cell size and cell cycle stage (Chapter 2). I learned that the extent of spermatogenesis displayed by a given population was influenced not by its average cell size as expected but rather by its cell cycle distribution.

This lack of a correlation between cell size and responsiveness to an induction cue was in direct conflict with the work of numerous other researchers. To try to understand the source of this discrepancy, I initiated a systematic examination of the manner in which cell size changed over time in a number of *T. weissflogii* isolates (Chapter 3). In the course of conducting this study I found evidence that the genetic composition of diatom populations varies over time. Thus, the characteristics of these populations can undergo frequent transformations, a feature which must be kept in mind as attempts are made to understand in more detail the physiology of these organisms.

I have also included in this thesis a study which describes cell cycle regulation in the procaryote, *Synechococcus* strain WH-8101, an organism which is unrelated to diatoms and in fact belongs to an entirely different kingdom (Appendix). This work is included here as an example of the fact that an understanding of the behavior of populations of cells requires an understanding of the behavior of the individual cells within the population. This philosophy, the "importance of the individuals", permeates each of the latter studies conducted with *T. weissflogii*.

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- Vaulot, D. & Chisholm, S. W. 1987. Flow cytometric analysis of spermatogenesis in the diatom *Thalassiosira weissflogii* (Bacillariophyceae). *J. Phycol.* 23:132-7.



## Chapter One

### Sexual Reproduction in Centric Diatoms





## Introduction

Diatoms (Bacillariophyta) are one of the most abundant groups of aquatic, eukaryotic microorganisms found in nature with approximately 100,000 described species dispersed among over 250 genera (Round and Crawford 1989). These unicellular algae are widely distributed among freshwater and marine habitats in both the plankton and the benthos, in fact "anywhere water drips, collects, or flows there is a diatom microbiota" (Round and Crawford 1989). Due to their dominance in oceanic upwelling zones and the regions of the continental shelves, diatoms are substantial contributors to world net primary productivity (Warner 1977). Moreover, these and other large phytoplankton have recently been hypothesized to play a crucial role in the new production of oligotrophic waters (Goldman 1988).

Despite their obvious ecological importance, however, many of the details of the physiology of these organisms remain mysterious. In an era when model systems such as the budding yeast, *Saccharomyces cerevisiae*, and the freshwater green alga, *Chlamydomonas reinhardtii*, can be genetically manipulated at will through techniques ranging from simple matings to intricate transformations (e.g. Herskowitz 1988, Boynton et al. 1988, Hall 1989), studies of the diatom life cycle have progressed little beyond the descriptive stage. And yet, the sexual cycle of diatoms may be unique among unicellular algae since the vegetative cells are

diploid and thus meiosis precedes gametic differentiation. Moreover, the induction of sexuality in diatoms has traditionally been thought to be linked with the obtainment of an appropriate cell size. If this proves to be true, the diatom life cycle may not only be unique among the phytoplankton but among the entire protist kingdom as well.

Relatively few life cycle studies have been conducted in the 13 years since Drebes's review of diatom sexuality was published (Drebes 1977b). This is perhaps due to the somewhat pernickety nature of diatoms; sexual events serendipitously observed once are frequently hard to replicate. Since 1977, however, research techniques available to scientists have blossomed; for example, painstaking microscopic observations are no longer the only means of detecting sexuality (e.g. Vulot and Chisholm 1987a, chapter 2). Thus, we may now be able to move beyond simple descriptions of the sexual process and begin to ask more detailed questions. For instance, an understanding of the mechanisms underlying the induction of sexual reproduction in the various diatom species may enable us to someday predict how changing environmental conditions will influence the extent of sexuality and thus the potential for genetic variability in diatom populations in the field.

## Historical Perspective

The diatom literature dating from nearly a hundred years ago overflows with wonderfully detailed descriptions and drawings of the life histories of some of the larger, more easily observed organisms (e.g. Yendo and Ikari 1918, Persidsky 1935, Gross 1937). Towards the end of the nineteenth century, researchers surmised that the unique structure of the diatom frustule imposed potential constraints on its life cycle. An assortment of cell sizes within a given species were generally observed but it was initially unclear whether cells gradually became smaller or larger over successive generations. The diatom frustule is composed of two rigid, unequally sized silica valves, the epitheca and the hypotheca, each attached to a series of silicious girdle bands. MacDonald (1869) and Pfitzer (Rao and Desikachary 1970) simultaneously surmised in what has become known as the MacDonald-Pfitzer hypothesis, that since the valves of the new frustules are created within the confines of the old, each mitotic division results in one daughter cell which is smaller than either her sister or her mother thus causing a gradual decrease in cell size over successive generations.

Some sixty years later, Geitler (1935) reviewed the details of the life cycles of a number of pennate diatom species and defined three distinctive categories of cells (still used today), characterized by their relative cell size. His first category consisted of the largest cells or post-auxospores, generally created

sexually, which were capable only of asexual reproduction. Size diminution of these vegetative cells created the second category, cells characterized by a smaller size range that were capable of both asexual and sexual reproduction. Individuals that passed through this size range without undergoing gametogenesis comprised the third category. These cells were no longer capable of sexual reproduction and simply continued to decrease in size; theoretically this cell line would eventually die. Thus by the early thirties, the foundation of what appear to be the unique features of diatoms had become well established: sexuality and perhaps even cell vigor are intimately associated with cell size.

As pennate diatoms became well characterized and were proven to possess isogamous or morphologically equivalent haploid gametes, arguments flourished as to the exact nature of sexual reproduction in centric diatoms. Flagellated cells which are now known to be sperm were observed (e. g. Gross 1937, Braarud 1939, Subrahmanyam 1946) but it was unclear exactly what role these cells played in the life cycle. For example, Gross (1937) concluded definitively that these "microspores" resulted from either an abnormal division process or the "occasional presence and reproduction in dead diatom cells of flagellates not necessarily of parasitic nature." Another fifteen years of disagreements had to pass before von Stosch (1950), through a detailed examination of field samples, firmly established that centric diatoms are characterized by oogamy, the formation of morphologically dissimilar sperm and eggs.

With the discovery that many diatoms require vitamin B<sub>12</sub>, the number of bacteria-free clones of diatoms maintained in culture increased dramatically during the fifties and sixties (Lewin and Guillard, 1963). In keeping with this trend, life cycle studies on centric diatoms moved from field observations to laboratory experiments.

#### Gametogenesis and Zygote Formation

The predominant mode of reproduction in diatoms is mitotic division. However, like many other eukaryotic microorganisms (Sager and Granick 1954, Esposito and Klapholz 1981, Pfiester and Anderson 1987) diatoms can exit the mitotic cycle and undergo gametogenesis. Since diatoms are diploid, meiosis precedes gametogenesis and thus the gametes may be morphologically and physiologically quite distinct from vegetative cells. Moreover, since diatoms are monoecious, each cell can differentiate into either male or female gametes. Drebes (1977b) presents a excellent summary of the numerous possible steps leading to the formation of the sperm, egg, and auxospore. Only a brief review will be presented here.

#### Spermatogenesis

The first indication of spermatogenesis is a series of specialized mitoses that creates within a single frustule, anywhere from one or two diploid spermatogonia as in *Skeletonema costatum* (Migita 1967b) to as many as 128 spermatogonia within a single *Coccolodiscus pavillardii* cell (Findlay 1969). These spermatogonia

may either swell and complete meiosis free of the mother frustule (e. g. French and Hargraves 1985, chapter 2) or else remain enclosed within the frustule until meiosis is completed (e.g. Findlay 1969). As no growth occurs between the successive mitotic and meiotic divisions, a male determined cell of *Lithodesmium undulatum*, for example, can complete four successive mitotic divisions and two meiotic divisions in as little as 12 hours (Manton et al. 1970), an interval comparable to the average doubling time of the vegetative cells of numerous species. The final outcome of these divisions is the creation of four unflagellated haploid sperm from each spermatogonium. Thus, a *C. pavillardii* cell, for example, can create as many as 512 sperm.

#### Oogenesis

Oogonia, unlike spermatocytes, are formed directly from the vegetative cell with no accompanying reductive mitoses. In the ensuing meiotic divisions, two eggs and two pycnotic or disintegrating nuclei, one egg and a polar body, or one egg and two pycnotic nuclei are formed depending on the species examined (Drebes 1977b). In certain species such as *Stephanopyxis palmeriana* (Drebes 1966) or *Leptocylindrus danicus* (French and Hargraves 1985), the females can be differentiated from vegetative cells since they are slightly elongated and can possess both an enlarged nucleus and an increased number of chloroplasts. In other species such as *Coscinodiscus cocinnus* (Holmes 1967) or *Chaetoceros diadema*

(Hargraves 1972), however, no conspicuous differences between females and vegetative cells are observed.

#### Auxospore Formation

A number of ingenious methods have been devised by different diatom species to facilitate the necessary union of the sperm and egg since the egg is generally still enclosed within her silica frustule. Often, the female bends slightly thus allowing the sperm to enter through a opening between the two valve halves (e.g. von Stosch 1950, Drebes 1966, French and Hargraves 1985). In other instances, the sperm apparently slithers through a bristle opening in the frustule (Drebes 1977b).

Upon fertilization, the developing diploid zygote or auxospore rapidly expands due to a sudden intake of water, squeezing the organelles into a thin layer of cytoplasm within the auxospore periphery (Hoops and Floyd 1979), and then escapes the confines of the mother frustule. At this stage the auxospore is surrounded by an organic wall frequently interspersed with siliceous scales (Crawford 1974, Hoops and Floyd 1979). The protoplasm eventually contracts from the wall and two new silica valves are laid down. Interestingly, an acytokinetic mitotic event (nuclear division without cytokinesis) accompanies the formation of each valve and thus two pycnotic nuclei are created (Drebes 1977b). The auxospore envelope finally ruptures and the newly enlarged "Erslingszelle" or initial cell is released. The relation between the size of the post-auxospore cell and the parent cell can vary (e.g. Rao 1971,



chapter 3), but the newly created cell may be as many as five times as large as either parent (Findlay 1969).

### Environmental Induction Signals

Diatoms can be induced to exit the mitotic cycle and undergo sexual reproduction in response to a suite of environmental signals including a sudden change in light intensity, temperature, nutrient status, or salinity (Table 1). The motivation behind many of the early studies on the induction of sexuality was either a manipulation of a given species through its various life cycle stages or a description of a serendipitously observed event rather than an examination of the interaction between a cell and its environment. The appropriate conditions for gametogenesis are often presented simply as recipes and the use of "old" cultures is frequently noted (e.g. Drebes 1966, Rao 1971, Drebes 1972, Hoops and Floyd 1979). Only rarely is the population response to an induction signal quantified, complicating any attempts to evaluate the relative "strengths" of the various induction signals. Thus, it is unclear whether or not the reported range of chemical and physical signals all induce sexual reproduction via a common mechanism.

#### Light Cues

The duration of the photoperiod is frequently emphasized as an important component of many induction "mixtures". The results obtained with one species, however, are often in direct conflict

with results reported for another species. For example, Steele (1965) found that only when *Stephanopyxis palmeriana* was maintained on a 16:8 L:D cycle (rather than a 12:12 or an 8:16 L:D cycle) would cultures form sexual cells. Similarly, Furnas (1985) reported that higher proportions of *Chaetoceros curvisetum* cultures underwent spermatogenesis when maintained on L:D cycles of 16:8 rather than either 12:12 or 8:16. This preference for sexual reproduction over asexual reproduction under long photoperiods is exactly the opposite of what Holmes (1966) found in his systematic study of *Coccinodiscus concinnus*. He exposed cultures to sixty-four different light and temperature combinations at L:D cycles of 16:8, 12:12, and 8:16. The extent of both spermatogenesis and auxospore formation was always enhanced under the short photoperiods. This sort of contradictory behavior unfortunately adds to an apparent intractability of diatom life cycle studies.

Perhaps a little of the mystery can be removed, however, based on recent results obtained with the marine diatom, *Thalassiosira weissflogii*. This diatom can be induced to undergo spermatogenesis when cells are transferred from saturating levels of continuous light to either dim light or darkness (chapter 2). Responsiveness to this induction signal, however, is a function of cell cycle stage. Only when cells are in early G<sub>1</sub> can they be induced to undergo spermatogenesis; cells further along in their cell cycle are unresponsive to the same induction cues and simply divide mitotically (chapter 2). If the response of *C. cocinnus*, *S.*

*palmeriana*, and *C. curvisetum* to a change in light conditions is also limited to cells in a particular stage of their cell cycle this may explain some of the conflicting photoperiod results.

In order to predict how the duration of the photoperiod can influence the number of cells induced to undergo gametogenesis, the manner in which light affects cell cycle progression must first be understood. Diatoms possess at least one and more often two light dependent regions, located in  $G_1$  and/or  $G_2$ , where continued cell cycle progression requires light (Vaulot et al. 1986, Olson et al. 1986, M. Brzezinski, pers. comm.). Light/dark cycles align the cells cycles of a culture depending on the length of the various cell cycle stages, the location and the duration of the light requiring segments, and the duration of the photoperiod (Vaulot and Chisholm 1987b). The proportion of a population that is induced to undergo gametogenesis under a given photoperiod is a function on the number of cells which are in the appropriate cell cycle stage during induction. If for example, the durations of the light requiring segments differ between *Chaetoceros curvisetum* and *Coscinodiscus concinnus*, the cell cycles of these two organisms will be differently aligned to a given light dark cycle and thus different proportions of cells will move through their inducible region during the induction signal and be triggered to undergo sexual reproduction.

### Nutrient Cues

Two diatoms that have been observed to undergo sexual reproduction in response to nutrient limitation are *Leptocylindrus danicus* (French and Hargraves 1985, 1986) and *Skeletonema costatum* (Davis et al. 1973). *S. costatum* cells can become sexual under silica limited continuous culture conditions. On the other hand, nearly one hundred percent of *L. danicus* populations are induced to undergo sexual reproduction in response to nitrogen limitation. The highly unusual feature of this system, however, is that immediately after zygote formation and cell enlargement, the auxospore always develops into a resting spore. Nitrogen deprivation apparently set into motion two new developmental pathways.

### Salinity Cues

One final species which holds promise for studying induction signals but has apparently not been examined since the late sixties and early seventies, is an estuarine species, *Cyclotella meneghiniana*. Sexuality in *C. meneghiniana* is triggered by a change in  $\text{Na}^+$  concentrations (Schultz and Trainor 1968, Schultz and Trainor 1970, Rao 1971). Interestingly, only male gametes were formed on a 15:9 L:D cycle whereas in continuous light, all stages in the sexual cycle were formed (Schultz and Trainor 1970) (see section on Sex Determination).

## Size and Sexuality

A remarkable feature of the diatom life cycle is that the ability to undergo sexual reproduction is apparently linked to the size of a cell (Drebes 1977b). Over successive generations the mean cell size of a population generally decreases (Rao and Desikachary 1970) and the standard deviation about this mean increases (for exceptions to this general rule of size reduction see for example, Rao and Desikachary 1970, Round 1972, Crawford 1980)). It is commonly observed that only when cells obtain a diameter less than about 30-40% of their species' maximum, can they undergo gametogenesis and subsequent auxospore formation (Drebes 1977b). This newly enlarged cell then proceeds along the asexual path until size once again permits sexuality and thus ensuring the periodic nature of diatom sexuality (Lewis 1984).

### Evidence

The ideal way to test the relationship between cell size and the ability to undergo gametogenesis is to examine genetically identical population of cells that differ only in their size distributions. This is nearly impossible to do, though, since diatoms are monoecious and thus sexual reproduction can occur in populations originating from a single cell. Von Stosch (1965) circumvented this problem by artificially eliciting size changes through nutritional and other cultural manipulations. He found that newly enlarged cells were not receptive to induction signals,

whereas cells which had been forced instead to decrease abruptly in size were receptive (Drebes 1977b). An examination of the response of cells which have been exposed to extreme conditions, however, is often problematic; the possibility of transient behavior unrelated to absolute cell size can not be eliminated. For example, enlarged cells were obtained by transferring cells to media essentially devoid of silica. Cells discarded their frustules to form "protoplasts" and only formed a new larger, but often misshapen frustule upon a return to silica replete conditions. It is unclear what other physiological changes may have accompanied these conditions.

An alternative method employed to examine the relationship between cell size and sexuality is to periodically remove aliquots from an isolate in the process of size diminution and thus expose populations with different size distributions to a given induction signal. Rao (1971) monitored cell size and susceptibility to a salinity induction signal in *Cyclotella meneghiniana* for 18 months and found that only cells less than approximately 38% of the maximum formed gametes. However, a single round sexual reproduction and auxospore formation did not always lead to the obtainment of the cells' maximum size. Instead, at least two size steps were observed; a portion of the cells that had undergone one round of sexual reproduction immediately underwent a second round to form even larger cells.

Similarly, Findlay (1969) followed a isolate of *Coscinodiscus pavillardii* for 15 months and found that sexuality occurred only in cells ranging from 20-63% of the maximum diameter. Although he did not mention the actual proportions of the populations that were induced as the average cell size of the isolate decreased, he did note that sexuality did not occur in all the cells within the inducible range; some cell lines continued dividing vegetatively and eventually died. This heterogeneous response of a population theoretically within the inducible size range has frequently been observed (e.g. Werner 1971) and obviously complicates any simple relationship between cell size and susceptibility to an induction trigger.

In a slight twist to this experimental design, I (chapter 2) isolated a number of *Thalassiosira weissflogii* populations displaying a range of size distributions. In contrast to expectations, no relationship was observed between the average size of the various isolates and the proportion of cells that were induced to undergo spermatogenesis in response to a change in light conditions. In a subsequent follow-up study, I monitored the changes in cell size in these various isolates during approximately two years of exponential growth in constant conditions (chapter 3). Each isolate displayed periodic increases and decreases in mean cell size as expected for populations alternating between asexual and sexual reproduction. However, the overall patterns of cell size change varied dramatically between the various populations despite

the fact that each culture was maintained under identical growth conditions. The only feature common to these patterns was the rate of decrease in mean cell size, a parameter which is apparently determined by the physical constraints of the frustule during asexual reproduction. The rest of the components, i.e., the timing of the sexual episodes, the rate of increase in average cell size and the size of the post-auxospore cells created during a sexual event could vary among isolates and even within a given isolate over time. Both the size at which a cell can undergo sexual reproduction and the size of the post-auxospore cells created during these sexual intervals could display genetic variability. I hypothesized that the extent of variation in the patterns of cell size change exhibited by the different populations resulted from the fact that the genetic composition of a population can vary over time.

#### Sex Determination

As mentioned previously, diatoms are monoecious and thus each cell can form either male or female gametes. Essentially nothing is known about how this decision making process is achieved in diatoms. The reported environmental cues often differ slightly between males and females (Steele 1965, Holmes 1966, Migita 1967b). For instance, in *Melosira moniliformis* (Migita 1967) and *Skeletonema costatum* (Migita 1967b), cells maintained in higher light intensities form females rather than males, whereas in *Stephanopyxis palmeriana* (Steele 1965), just the opposite is true. Cell size has also been



observed to influence the sex of a cell. For example, in *Cyclotella meneghiniana* (Rao 1971), cells within the low end of the inducible size spectrum form males whereas the larger inducible cells form females. As with environmental cues, exactly the opposite response can be found in other diatoms; *Gaetoceros diadema* forms females from small cells and males from larger cells (Hargraves 1972).

#### Future Studies

Our understanding of those factors which allow a cell to be exit the mitotic cycle and undergo sexual reproduction is still quite limited. Although the obtainment of an appropriate cell size is strongly correlated with an ability to respond to environmental cues (e.g. Drebes 1977b), this permissive size range can vary among different isolates of a single species (chapter 3). Moreover, not all the cells within an appropriate size range respond to induction triggers (e.g. Findlay 1969, Werner 1971). For instance, I found that only cells in the  $G_1$  stage of their cell cycle can initiate spermatogenesis in response to a change in light intensities (chapter 2), (see section on Environmental Induction Signals). However, even within this  $G_1$  category, a portion of the cells may still not respond to the induction signal. Other parameters besides cell size or even cell cycle stage must differentiate small inducible cells from small uninducible ones. Could this also be what

differentiates post-auxospores from inducible cells? In Rao's study (1971), what permitted cells to form sequential sets of auxospores?

Many of these types of questions require an understanding of the underlying mechanisms of the induction of sexual reproduction. However, as is apparent from an inspection of Table 1, a common component of sexual cues is some form of environmental stress. This fact makes an assessment of the processes underlying the environmental induction of gametogenesis quite complicated since the cues generally elicit a host of physiological responses, most of which are unrelated to the induction of gametogenesis (e.g. Goodenough 1983). Assuming, however, that certain cells for at least a portion of their life cycle are unresponsive to induction signals (see section on Size Control of Sexuality), details of the physiological changes preceding the onset of gametogenesis can be compared between inducible and these unresponsive cells. Those changes which are unrelated to the induction of gametogenesis will be common to both populations. Early changes found only in the responsive population should be associated with induction.

#### CONCLUSIONS

Diatoms are apparently unique among the phytoplankton because they are diploid and because they display a gradual decrease in cell size over successive generations. Numerous studies have linked the obtainment of an appropriate cell size with susceptibility to these

induction signals. The fact that often only a portion of the cells within an inducible size range responds to a given signal indicates that other factors besides cell size must control sexuality. It is now clear that the cell cycle distribution of a population upon induction can influence the proportion of cells that can respond to an induction signal. In addition, both the size at which a cell can undergo sexual reproduction and the maximum size of a post auxospore cell can display genetic variability.

Since diatoms are diploid, genetic recombination during sexual reproduction will generate variability in the genotypes and perhaps even the phenotypes of the individual cells within a given population over time. This means that many aspects of the behavior of diatoms may be quite variable, a feature of diatom populations which must be kept in mind as attempts are made to understand the details of the physiology of these organisms.

Table 1. Summary of sexual induction signals reported for a number of centric diatoms.

Genus	Species	Induction Signal	Sexual Cells Formed <sup>a</sup>	Reference
<i>Coscinodiscus</i>	<i>cocinnus</i>	light	M/A	Holmes 1966
		temp		
	<i>pavillardii</i>	salinity		Findlay 1969
	<i>asteromphalus</i>	temp.	M	Verner 1971
		light		
<i>Melosira</i>	<i>moniliformis</i>	light (strong)	F	Nigita 1967a
		light (weak)	M	
<i>Stephanopyxis</i>	<i>palmeriana</i>	light		Steele 1965
		(L:D, 16:8,	M	
		high intensity)		
		(L:D, 16:8	A	
		low intensity)		
<i>Thalassiosira</i>	<i>weissflogii</i>	light (L:D, 14:10)		Drebes 1966
		temp		
		light/dark	M	Vaulot & Chisholm 1987
		subsaturating	M	Armbrust et al. 1990
<i>Skeletonema</i>	<i>costatum</i>	light		
		silica	M/F/A	Nigita 1967b
			M/F/A	Davis et al. 1973

Table 1. cont.

Genus	Species	Induction Signal	Sexual Cells Formed <sup>*</sup>	Reference
Pytilum	brightwellii	manganese	M/A	Steele 1965
Lithodesmium	undulatum	fresh medium, temp. light		e.g. Manton & von Stosch 1966
Cyclotella	meneghiniana	Na <sup>+</sup> (L:D, 15:9)	M	Schultz & Trainor 1970
		Na <sup>+</sup> (LL)	A	
		Na <sup>+</sup>	M/F/A	Schultz & Trainor 1968
		salinity.	M/F/A	Rao 1971
		"old" cultures		
	cryptica	salinity	M/F/A	Schultz & Trainor 1970
Chaetoceros	curvisetus	light/dark	M	Furnas 1985
	diadema	nitrogen	M/F/A	French & Hargraves 1985
	simplex	light	A	Sato 1967
Bacteriastrium	hyalinum	temp. light		Drebes 1972
		"old" cultures	M/F/A	Drebes 1967
Leptocylindrus	danicus	nitrogen	M/F/A	French & Hargraves 1985
Attheya	decora	light	M/F/A	Drebes 1977a

<sup>\*</sup> M-male gametes, F-female gametes, A-anisospores.

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## Chapter 2

### Induction of Spermatogenesis in a Centric Diatom: The Role of Light and the Cell Cycle



# ABSTRACT

The centric diatom, *Thalassiosira weissflogii* Grun., can be induced to undergo spermatogenesis by exposing cells maintained at saturating levels of continuous light to either dim light or darkness. Using flow cytometry to determine the relative DNA and chlorophyll content per cell, the number of cells within a population which responded to an induction signal was measured. From 0 to over 90% of a population could differentiate into male gametes depending upon both the induction trigger and the population examined, regardless of cell size.

Through the use of synchronized cultures, we demonstrated that responsiveness to an induction trigger is a function of cell cycle stage; cells in early G<sub>1</sub> are not yet committed to complete mitosis and can be induced to form male gametes, whereas cells further along in their cell cycle are unresponsive to these same cues. A simple model combining the influence of light on the mitotic cell cycle and on the induction of spermatogenesis has been developed to explain the observed diversity in population responses to changes in light conditions.

Key index words: cell cycle, diatom, light, sexual reproduction

Diatoms, like all unicellular algae, reproduce predominantly via mitotic division. The diatom frustule is generally unable to expand apically during these mitotic divisions resulting in a decrease in the mean cell size of a population and an increase in the standard deviation about this mean over successive generations (MacDonald 1869, Rao and Desikachary 1970, Round 1972). The most common manner of escaping this trend of diminishing cell size is through sexual reproduction. It is commonly believed that once cells reach a diameter less than 30-40% of their species' maximum diameter, they can be induced by an environmental trigger to exit the mitotic cycle and undergo gametogenesis (e.g. Drebes 1977). The resulting male and female haploid gametes combine to create a diploid auxospore which enlarges outside the confines of the parental frustule to produce a cell many times larger than either parent (e.g. Migita 1967, Rao 1971). This newly created cell then proceeds along the asexual pathway until an appropriate trigger once again elicits gametogenesis.

Although the developmental pathways of both spermatogenesis and oogenesis have been well documented in a number of diatom species (von Stosch 1950, Manton et al. 1969a,b, 1970a,b, Drebes 1972.), the underlying mechanisms for triggering these pathways remain elusive. A suite of factors including light (e.g. Drebes 1966, Furnas 1985, Vaultot and Chisholm 1987), nutrients (e.g. Davis 1973, French and Hargraves 1985), salinity (e.g. Schultz and Trainor 1968) and temperature shifts (e.g., Holmes, 1966) have all been

implicated in initiating this switch from asexual to sexual reproduction.

We recently discovered a means of inducing the centric diatom *Thalassiosira weissflogii* to undergo spermatogenesis (Vaulot and Chisholm 1987). Upon the return of a dark arrested population to continuous light, a portion of the cells differentiated into male gametes. The majority of the population, however, apparently "ignored" this cue and continued dividing asexually. This heterogeneous response of diatom populations to an environmental signal has been observed frequently and is often attributed to the non-responsiveness of large cells. However, even within a purportedly appropriate size range, often only a fraction of a culture responds to the cue (e.g. Findlay 1969, Werner 1971). This observation complicates any simple relationship between relative cell size and susceptibility to an induction trigger and suggests that additional factors must underlie the heterogeneous responses of populations. The goal of our study with *T. weissflogii* was to explore responsiveness to an induction signal as a function of both cell size and cell cycle stage.

#### MATERIALS AND METHODS

Clonal isolates of *T. weissflogii* clone Actin (from the Culture Collection of Marine Phytoplankton, Bigelow Laboratories for Ocean Sciences) were obtained by isolating colonies from 2% agar



plates and transferring them to f/2 enriched seawater media (Guillard 1975) with nitrate as the nitrogen source. Cultures of these isolates were obtained whose volumes ranged from approximately 600 to 1200  $\mu\text{m}^3$  (as determined with a Coulter Electronics Model Z<sub>m</sub>/C256 electronic particle counter). Although Coulter values are determined by assuming that each particle is a sphere, these diameters accurately predicted the apical diameter of cells measured independently with a microscope. Each experiment began with semi-continuous cultures of the various isolates maintained at 20°C in saturating levels of continuous illumination (cool-white fluorescent). In all instances, unless specifically stated, the photon flux density (PFD) was constant at 250  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$  as measured with a Biospherical Instruments model QSL100 irradiance meter. A Turner fluorometer was used to monitor daily changes in the *in vivo* fluorescence of the cultures to determine growth rates (Brand et al. 1981). During the sorting experiments, cell number was determined microscopically with a Fuchs/Rosenthal hemocytometer. A minimum of 300 cells were counted for each sample. For all other experiments, cell number and volume distributions were determined with the Coulter Counter.

To determine DNA content per cell, live samples were stained with 10  $\mu\text{g}\cdot\text{ml}^{-1}$  of the DNA fluorochrome Hoechst 33342 (Calbiochem, La Jolla, CA) for at least 10 min and relative DNA and chlorophyll fluorescence were measured with either a microscope based (Olson et al. 1983, Frankel et al. 1988) or a Coulter Epics V flow cytometer.

In both systems, UV excitation was 365 nm and chlorophyll fluorescence emission was measured between 660-700 nm. DNA fluorescence emission (Hoescht 33342) was measured between 430-470 nm on the microscope based flow cytometer and between 418-530 nm on the Epics V. All sorting experiments were conducted using the sorting capabilities of the Epics V.

Because some of the stages in the formation of male gametes are not enclosed within a frustule and are therefore particularly sensitive to the fixation process normally used for DNA analyses (Olson et al. 1983), only live samples were analyzed. This also allowed us to measure chlorophyll fluorescence per cell which increased our ability to discriminate between the various sexual stages. Because the coefficient of variation of DNA measurements is greatly increased in live cells (Olson et al. 1983), however, we could only divide the cell cycle of the vegetative cells into the G<sub>1</sub> and G<sub>2</sub> stages which are assumed to represent a DNA content of 2c and 4c, respectively (where c represents chromosome complement).

A known quantity of preserved nuclei from calf thymocytes (Ortho Diagnostic Systems) was added to each sample to act both as an internal staining standard and to permit a calculation of the density of cells within a given subpopulation. At least 30,000 cells were analyzed for each treatment at a minimum of two separate time points.

The flow cytometric data were stored as two parameter histograms of correlated DNA and chlorophyll fluorescence of either

64 X 64 (Epics V) or 128 X 128 (microscope based) channel resolution. The data generated with either instrument were transferred to an IBM 9000 computer for detailed analysis (Vaulot 1986).

## RESULTS

*Detection and analysis of spermatogenesis.* When *T. weissflogii* populations were maintained in saturating intensities of continuous light, a typical flow cytometric "signature" of a population revealed that the majority of the cells were located in either the  $G_1$  or  $G_2$  portion of the cell cycle (Fig. 1A); few if any male gametes were produced in these cultures, as shown by a general absence of cells with anything other than two or four complements of DNA. Cells transferred from continuous light to prolonged darkness did not undergo spermatogenesis in the dark, again as indicated by the presence of cells in only the  $G_1$  or  $G_2$  phase of the cell cycle (Fig. 1B). An interruption of growth in continuous light with 12 h of darkness, however, induced a portion of a population to exit the mitotic cell cycle and undergo spermatogenesis (Fig. 1 C-H).

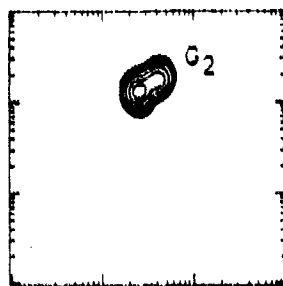
At the end of the 12 h dark interval, the induced and non-induced cells within the population were indistinguishable both morphologically and flow cytometrically (cf. Fig. 1A, C). After 3 h of light exposure, however, a low chlorophyll subpopulation developed creating a distortion in the flow cytometric signature of the vegetative cells (Fig. 1D). Two h later, two subpopulations emerged with lower chlorophyll fluorescence than either the  $G_1$  or  $G_2$

cells; one consisted of cells with a  $G_2$  complement of DNA (4c) and the other consisted of cells with twice this amount of DNA (8c) (Fig. 1E). Soon thereafter, the new 4c and 8c subpopulations were well distinguished from the vegetative cells by virtue of the increased chlorophyll fluorescence of the vegetative cells (resulting from the return to continuous light) (Fig. 1F). Twelve h after the return to the light (Fig. 1G), two more subpopulations developed with 2c and 1c DNA contents. By this time, the flux of cells from the vegetative to the low chlorophyll populations was complete and the low chlorophyll cells simply flowed from the 8c to the 1c population. Approximately 30 h after the return to continuous light, the majority of the low chlorophyll cells contained a 1c amount of DNA (Fig. 1H).

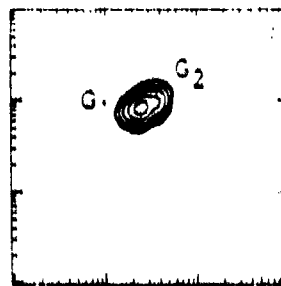
Morphological identification of these various subpopulations was obtained using the sorting capabilities of the Epics V flow cytometer. The sorted cells were examined microscopically (Fig. 2) and were found to fit the descriptions in the literature of the male sexual stages (e.g. Drebes 1977). The 8c population corresponded to spermatogonangia containing two 4c spermatocytes within a single frustule (Fig. 2B). The 4c population consisted of naked spermatocytes free of the frustule (Fig. 2C) and the 2c population were small flagellated cells (Fig. 2D). The 1c cells, which are the culmination of this process, were motile sperm (Fig. 2E). The developmental pathway of *T. weissflogii* adhered to the relatively common hologenous type described by Drebes (1977), since each

Fig. 1. Flow cytometric signatures of relative DNA and chlorophyll fluorescence per cell measured under three different light regimes. A) An exponentially growing population maintained in continuous light and B) transferred to an extended interval of darkness (94.5 h). C-H) Time course of gametic differentiation in a second population that had been transferred to the dark for 12 h and returned to continuous light at t=0 h. The contour levels represent 0.008, 0.012, 0.020, 0.036, 0.064, 0.108, and 0.158 % of the total cell number.

Relative Chlorophyll Fluorescence



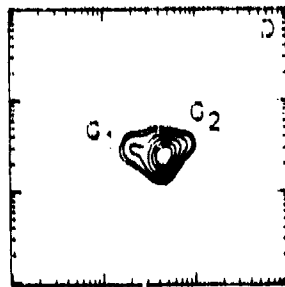
continuous light



prolonged darkness



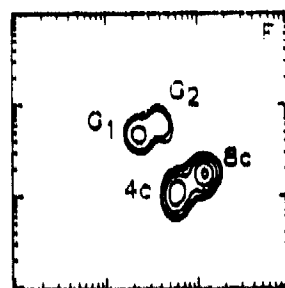
t=0 hrs



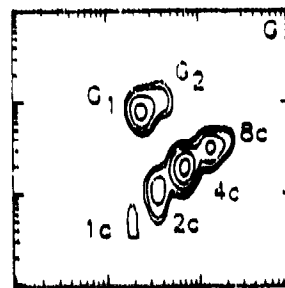
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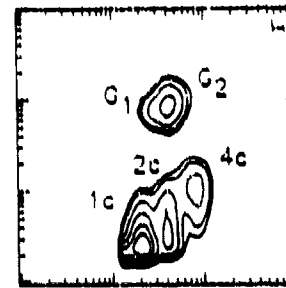
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t=9.5 hrs



t=12 hrs

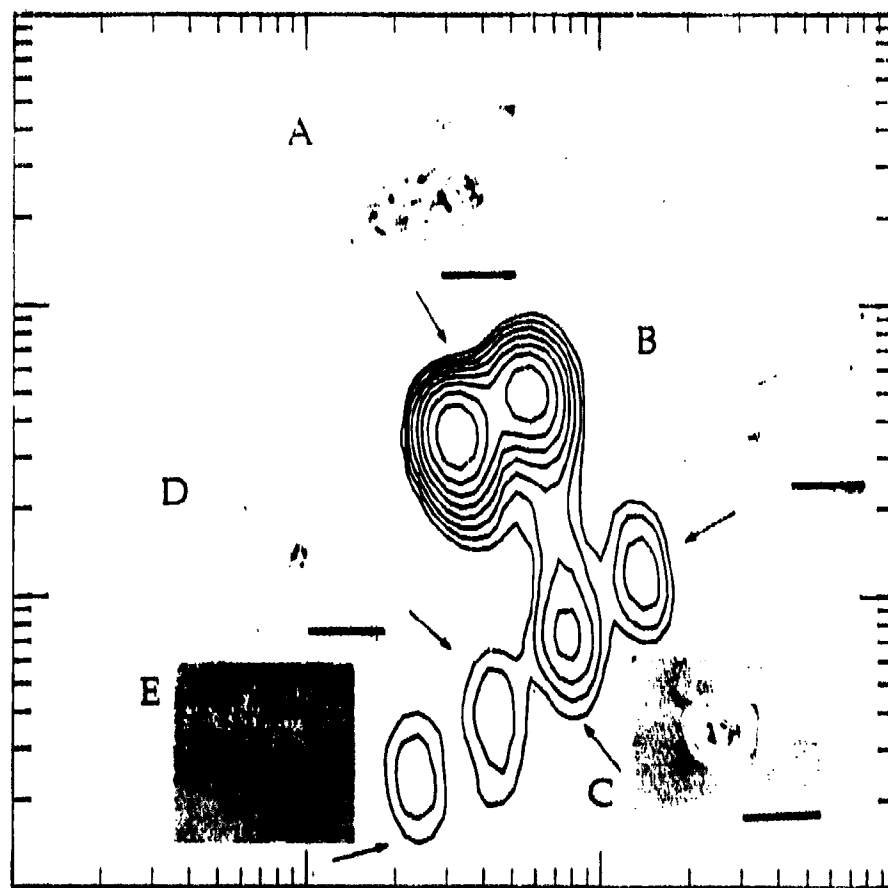


t=34 hrs

Relative DNA Fluorescence

Fig. 2. Flow cytometric signature of an induced population containing all four of the stages in the formation of male gametes. The contour levels represent 28, 42, 63, 95, 142, 213, 320 and 480 cells. A) A vegetative cell, B) an 8c spermatogonangium containing two 4c spermatocytes, C) a 4c spermatocyte remaining inside one half of the mother frustule, D) a small 2c cell, and E) a 1c sperm. Scale bars = 10  $\mu$ m.

Relative Chlorophyll Fluorescence



Relative DNA Fluorescence



meiotic division was accompanied by cytokinesis. This same general time course of differentiation was observed regardless of the *T. weissflogii* isolate examined and is similar to that observed by Manton et al. (1970b) for *Lithodesmium undulatum*.

With this understanding, we were able to calculate the number of cells in a given population that was originally induced to exit the mitotic cell cycle. At various times after the release of the population into the light, the number of cells in each of the sexual stages was determined (Fig. 3A), and the number of vegetative cells that had therefore been triggered to differentiate into gametes was calculated according to the following equations:

$$t < 8 \text{ h after release into the light:} \quad (1)$$

$$V = (A + B + C/4 + D/8)$$

$$t > 8 \text{ h after release into the light:} \quad (2)$$

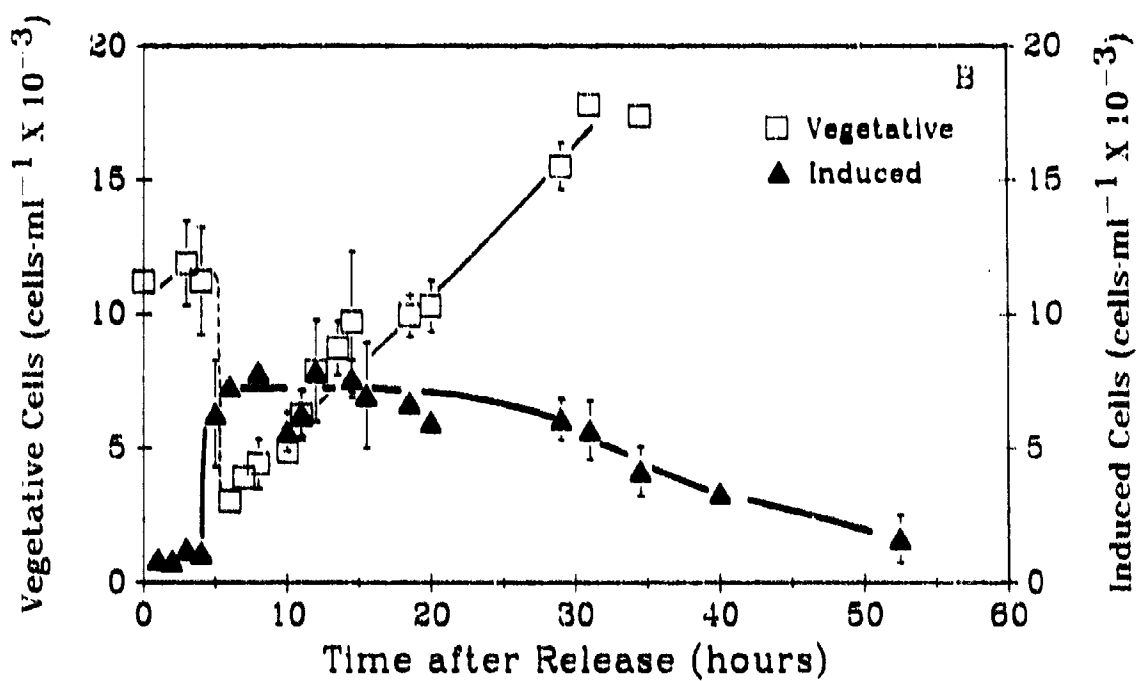
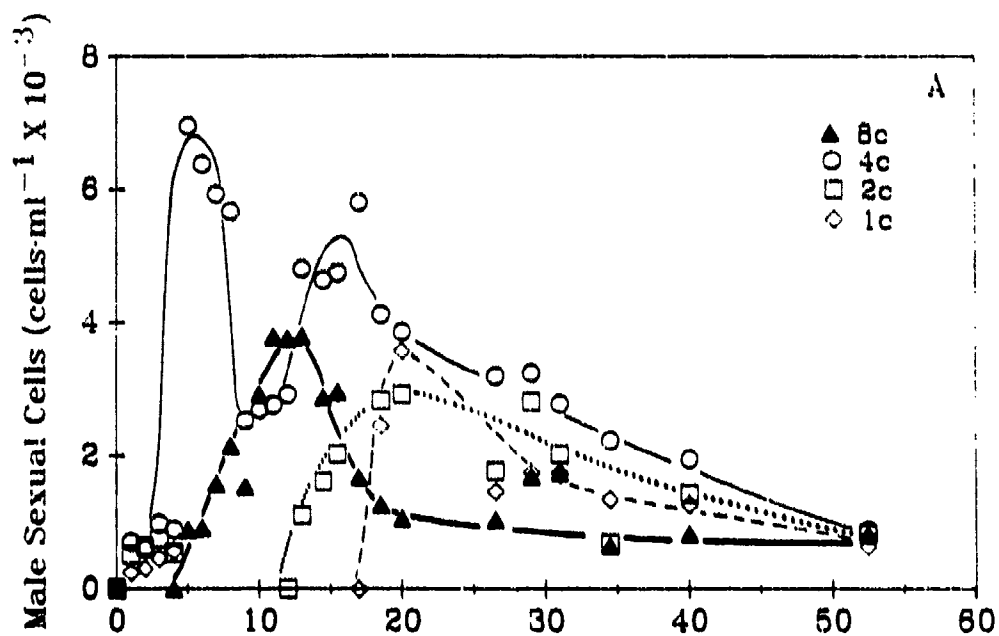
$$V = (A + B/2 + C/4 + D/8)$$

where V = the number of vegetative cells that have exited the cell cycle and are committed to undergo spermatogenesis; A, B, C, and D = the number of 8c, 4c, 2c, and 1c cells, respectively, present within the population at a given time. These two equations differed slightly because the 4c flow cytometry signature represented two different cell types as exemplified by the bimodal distribution in the number of 4c cells over time (Fig. 3A). Initially, the 4c cell was derived directly from an induced parent cell and remained enclosed within the frustule (Eq. 1). A peak in the number of these

cells occurred approximately 6-8 h into the differentiation process (Fig. 3A), at which point the peak declined rapidly as premeiotic DNA synthesis was initiated and the 8c cells were formed. A few hours later, the number of 4c cells again increased as the two naked 4c spermatocytes were released from each spermatogonangium. Thus, the number of these 4c cells reflected one half the number of originally induced cells (Eq. 2). The timing of this transition from 4c cells within the frustule to naked spermatocytes varied only slightly between experiments. However, to ensure that the plateau in the number of induced cells was obtained (Fig. 3B), a minimum of two time points were always analyzed for each treatment.

Through this analysis the proportion of cells that were not receptive to the induction cue was estimated (Fig. 3B). Approximately 3-5 h after the dark induced population was returned to the light, the number of vegetative cells suddenly declined as a portion of the population exited the mitotic cell cycle and initiated spermatogenesis (Fig. 3B). The entrance into gamete formation was fairly synchronous, occurring within a 3-5 h window. After the initial decline in vegetative cell number, asexual division continued at approximately the same rate as before induction (doubling time=10.4 h, Fig. 3B). A plateau in the number of induced cells occurred between 10 and 18 h after the population was returned to the light. The number of induced cells then declined and the sperm began to disappear. The male sexual cells had disappeared from the media approximately 50 h after the dark

Fig. 3. Mitotic division and the formation of the male sexual stages as a function of time in a population induced to undergo spermatogenesis with twelve h of darkness (as described in Fig. 1).  $t=0$  h is the time of the release of the population into continuous light. A) The mean density (of replicate flasks) of each of the stages in the formation of male gametes. B) The number of cells in the original population which continued reproducing asexually ( $\square$ ) and the number of cells which formed male gametes ( $\Delta$ ). Error bars larger than the symbol size are shown and represent the standard deviation of replicate flasks.



induced population was returned to continuous light. Presumably the sperm simply disintegrated, as no sign of auxospore formation was observed during or for approximately one week after this interval.

*Relationship between cell size and responsiveness to a dark induction signal.* Isolates with mean Coulter volumes ranging from 600 to 1200  $\mu\text{m}^3$  (see Methods) were exposed to 12 h of darkness, and the percentage of each population which underwent spermatogenesis was determined (Fig. 4A). No relationship between the percentage of cells which formed gametes and the mean Coulter volume of a culture was observed. Cultures representing widely different size distributions (Fig. 4B) yielded a comparable percentage of induced cells whereas other cultures representing nearly identical size distributions (Fig. 4C) resulted in very different responses. We concluded that in the isolates we examined, the induction of spermatogenesis was unrelated to cell size. We should note, however, that the average Coulter volume of the largest *T. weissflogii* isolate that we have observed in culture is 2000  $\mu\text{m}^3$  (Armbrust and Chisholm unpubl. data), thus many of the cultures used in this experiment contained cells that were less than the maximum size. Very large cells may be unresponsive to induction signals.

*Influence of dark interval duration on spermatogenesis.* We examined whether the duration of dark exposure influenced the proportion of a culture that was induced to undergo spermatogenesis

by subjecting populations to a range of different light/dark regimes. A culture maintained in continuous light was transferred to the dark for a total of 16 h and subsamples were removed every 2 h and returned to continuous illumination. The resulting percentage of cells induced to undergo spermatogenesis was then determined for each population. Because cell division continues for some time in the dark, the proportion of the population induced to undergo spermatogenesis was normalized to the number of cells present at the end of each dark interval.

Cell division continued for approximately 10 h in the dark (Fig. 5A) until all the cells were blocked in either G<sub>1</sub> or G<sub>2</sub> (Fig. 1C; Vaultot et al. 1986). Interestingly, the percentage of cells that underwent spermatogenesis also increased as the duration of the dark interval was lengthened to 10 h (Fig. 5B), indicating that cells continued to be induced during the dark period and not simply upon a transfer to darkness. A strong positive correlation was observed between the number of cells that divided during the dark interval and the number of cells that subsequently formed gametes in the light (Fig. 5C). Once cell division stopped in the dark no more cells were induced to undergo spermatogenesis thus suggesting that cell cycle progression during the dark interval is a necessary component of induction.

*The role of light intensity.* Morphological differentiation did not begin in any of the isolates examined until the dark induced

Fig. 4. The influence of cell size on the induction of spermatogenesis. A) Maximum percentage of populations induced to undergo spermatogenesis as a function of the mean Coulter volume of the population. Each population was induced to undergo spermatogenesis with 12 h of darkness. Closed circles and arrows indicate cultures examined in B and C. Error bars larger than the symbol size are shown and represent the standard error of triplicate flasks. B) The Coulter volume distributions of the two isolates marked "B" in A that displayed a comparable level of induction. Sixty-six percent of a population with a mean Coulter volume of  $660 \mu\text{m}^3$  underwent spermatogenesis; a similar percentage of the population with a much greater mean Coulter volume was induced to form male gametes. C) The Coulter volume distribution of the isolates marked "C" in A. For these two isolates, 7% versus 55% of the populations underwent spermatogenesis even though they displayed the same size distribution.

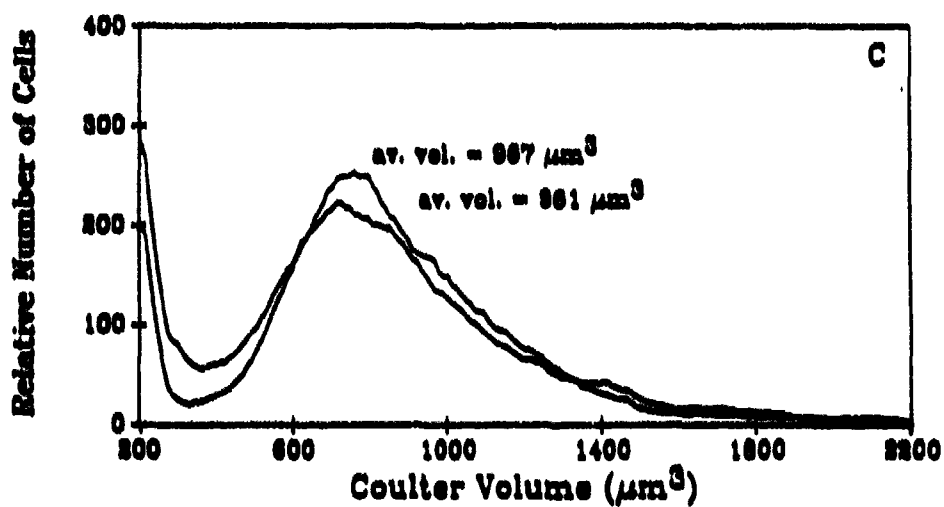
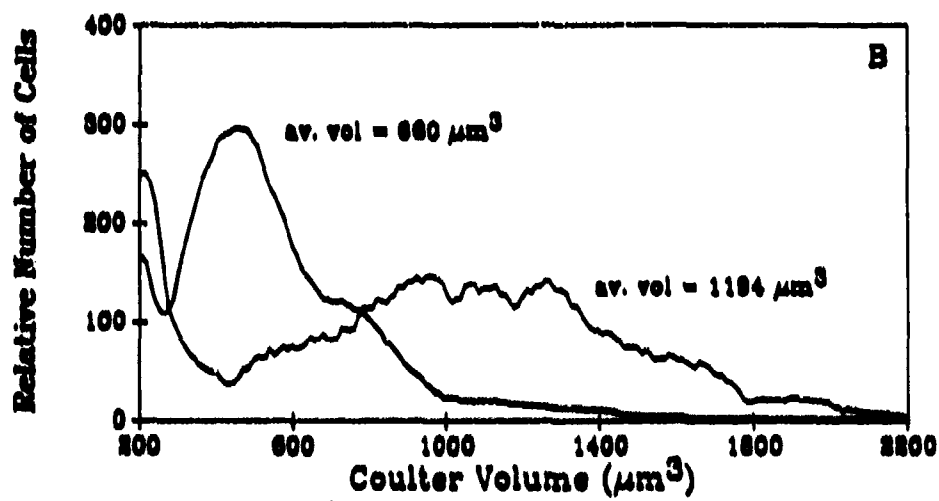
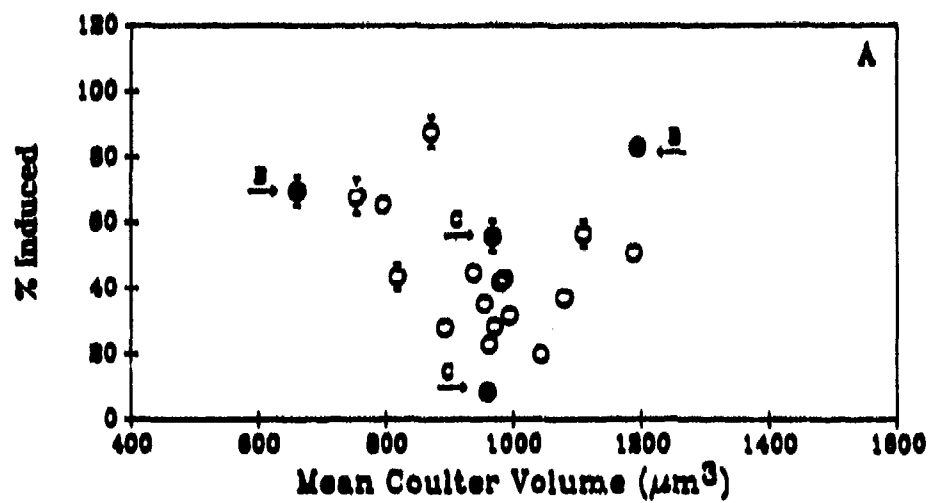
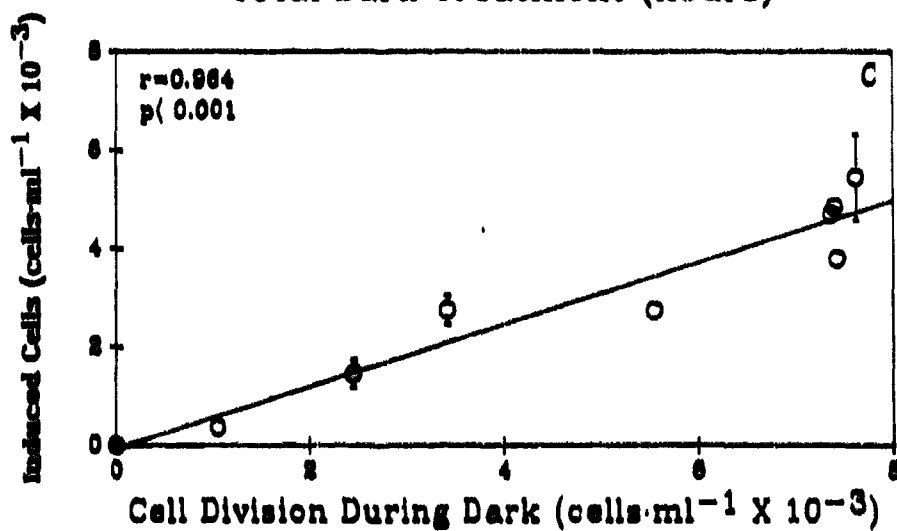
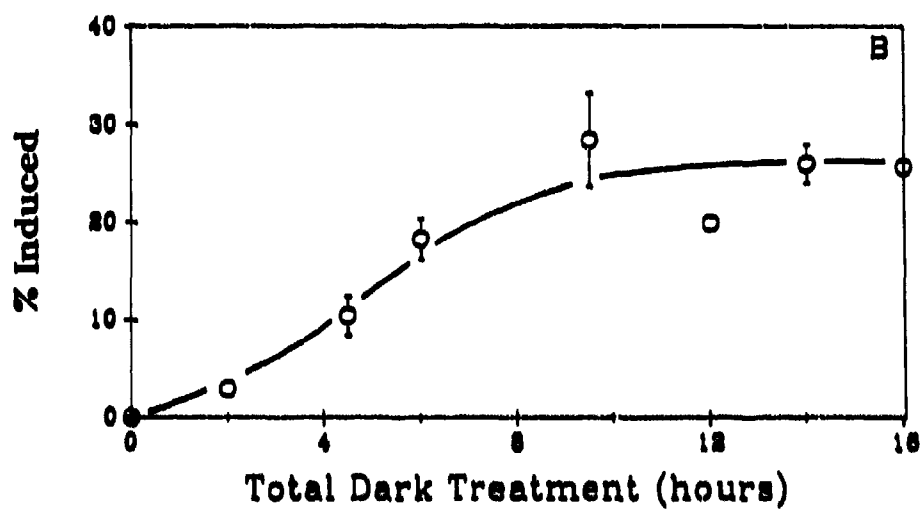
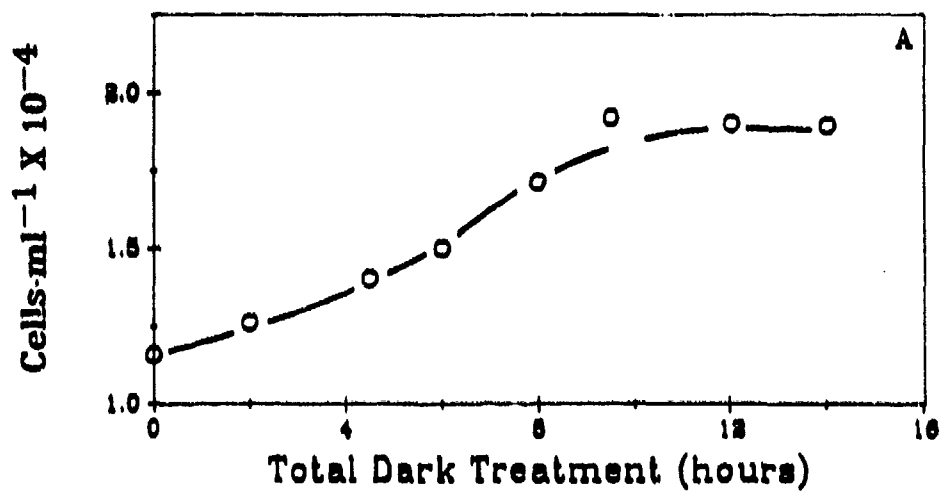




Fig. 5. Induction of spermatogenesis within a single culture as a function of the duration of dark treatment. A) Cell number as a function of time in the dark. The population was transferred from continuous light to darkness at  $t=0$  h, and replicate subsamples were removed from the dark every 2 h and returned to continuous light. B) Maximum percentage of cells induced to undergo spermatogenesis as a function of the amount of time in the dark. The percentage is calculated using the "plateau" region of the induction curve (cf. Fig. 3B). C) Number of cells which divided during the dark interval versus the number of cells which formed gametes after the population was returned to the light. Error bars larger than the symbol size are shown and represent the standard deviation of replicate cultures.



population was returned to the light (Fig. 1) suggesting that light functioned either as an additional signal (Kuhlemeier et al, 1987) which allowed differentiation to proceed, or as a necessary energy source for continued development. To distinguish between these two hypotheses, cells were maintained in  $250 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$  of continuous illumination (saturating for growth, see below), placed in the dark for 12 h, and shifted to a variety of saturating (Fig. 6A) and subsaturating (Fig. 6B) PFDs. The time course of gamete formation was then determined for each light regime.

To our surprise, we found that a much larger percentage of cells were induced to undergo spermatogenesis when cultures were shifted from darkness to subsaturating rather than saturating PFDs (Fig. 6A, B). Moreover, the dark induced populations that were shifted to a variety of saturating PFDs each formed approximately the same maximum percentage of gametes (Fig. 6A). In contrast, the percentage of cells that formed gametes under subsaturating PFDs was a function of PFD; the higher the PFD (up to saturating levels), the greater the percentage of cells which underwent spermatogenesis (Fig. 6B). Subsaturating light itself appeared to function as an induction signal.

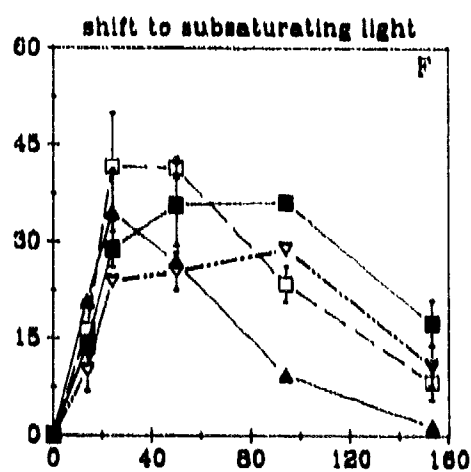
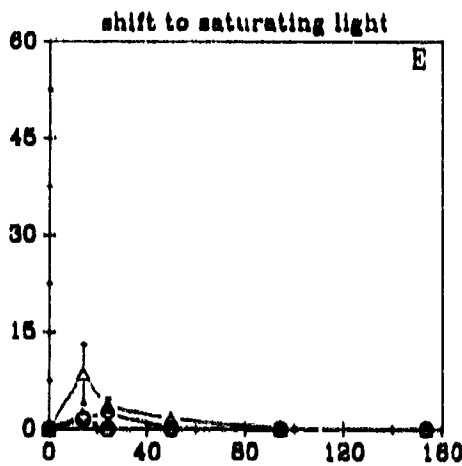
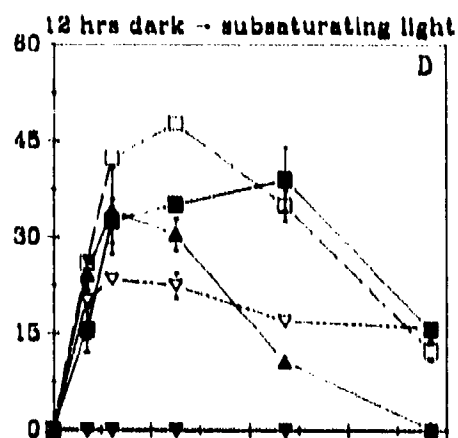
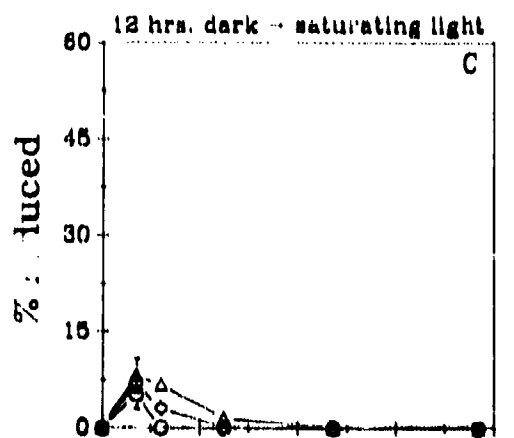
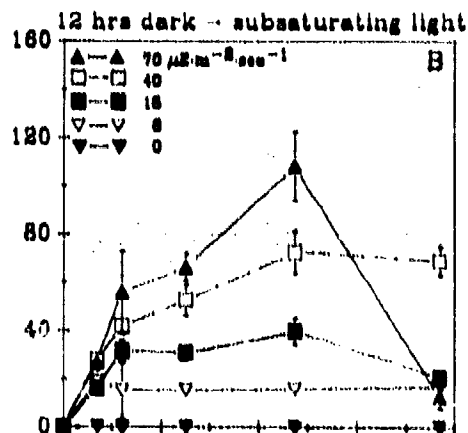
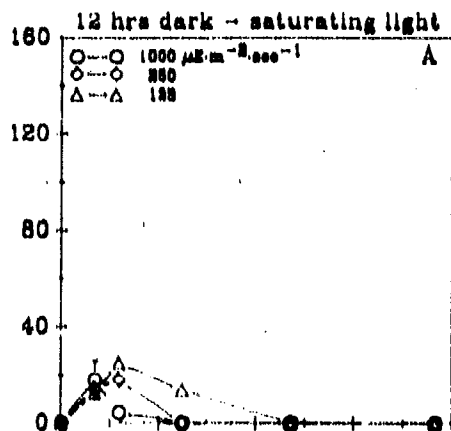
To test this possibility, populations that had been placed in the dark for twelve h and then shifted to a range of PFDs (Fig. 6C, D) were compared with populations shifted directly to these same PFDs without the preceding twelve h of darkness (Fig. 6E, F). Cells shifted directly from saturating to subsaturating PFDs of less than

$100 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$  were induced to undergo spermatogenesis; a transfer to darkness was not a necessary step (Fig. 6F). Moreover, the magnitude and the time course of the response of each population shifted to subsaturating PFDs was almost identical regardless of whether or not darkness was included as a part of the induction scheme (cf. Figs. 6D, F). In each instance, a peak in the percentage of the population induced to undergo spermatogenesis was observed approximately one day after the cultures were shifted.

The maximum percentage of a population induced to undergo spermatogenesis was many times higher in the populations shifted to subsaturating rather than saturating PFDs (Fig. 7) regardless of whether or not dark induction was included. The percentage of cells induced to form gametes increased as the PFD of the shift and thus the growth rate in these new conditions increased (Fig. 7, inset) until saturating PFDs were approached. At this point a drastic decrease was observed in the proportion of the population that underwent spermatogenesis (Fig. 7).

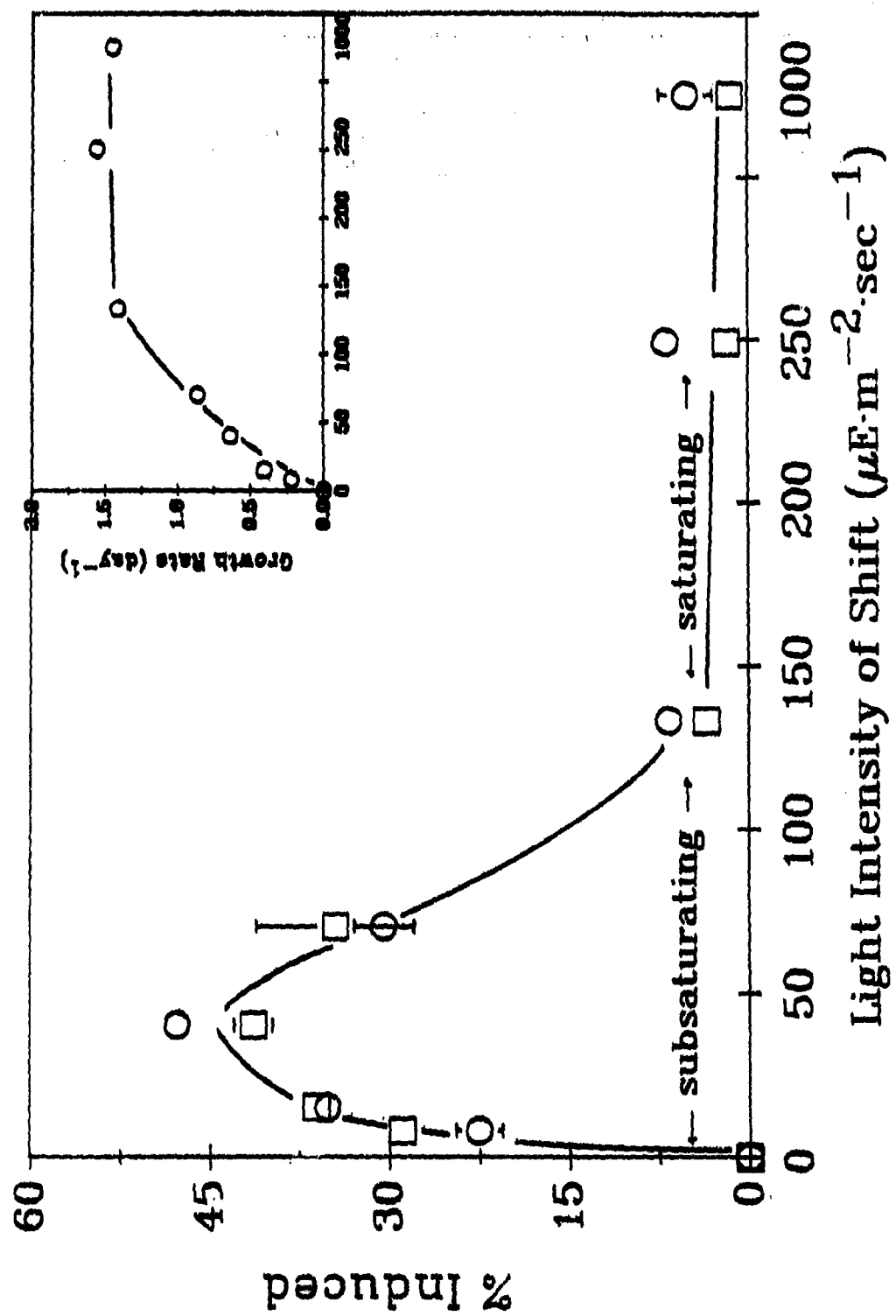
*The role of the cell cycle.* The dark induction and light intensity experiments suggested that a transfer to dim light or darkness functioned as an induction signal only when cells moved into or through a particular portion of their cell cycle. Populations of cells representing distinct cell cycle stages were necessary to test this hypothesis. Since *T. weissflogii* cells arrest in both  $G_1$  and  $G_2$  in the dark (Vaulot et al. 1986), in

Fig. 6. Percentages of cells induced to undergo spermatogenesis as a function of time under a range of different light regimes. Populations maintained in  $250 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$  of light were placed in the dark for twelve h and then shifted to either (A) saturating or (B) subsaturating photon flux densities. The number of cells induced to undergo spermatogenesis was normalized to the number of cells present at the end of the dark interval. C, D) Since subsaturating light can function as an induction signal, the same data as in A and B were normalized to the number of cells present at the time the culture was sampled (note the change in scale) to permit a direct comparison with populations transferred directly to the various saturating (E) and subsaturating (F) PFDs without the accompanying 12 h of darkness. Error bars larger than the symbol size are shown and represent the standard deviation of replicate flasks.



Time after Release (hours)

Fig. 7. The maximum proportion of each population described in Fig. 6C, D, E, and F which underwent spermatogenesis when shifted directly to the various light intensities ( $\square$ ) or first placed in the dark for 12 h and then shifted ( $\circ$ ). Error bars larger than the symbol size are shown and represent the standard deviation of replicate flasks. Inset: Steady state growth rate of *T. weissflogii* as a function of light intensity.





contrast to many other phytoplankton (Nelson and Brand 1979), synchronized cultures could not be obtained by maintaining populations on a light/dark cycle. Instead, populations representing a range of cell cycle distributions were obtained using the sorting capabilities of the Epics V. Because chlorophyll content appeared to vary with cell age (Fig. 1A), a number of subpopulations were sorted based on their chlorophyll fluorescence (Fig. 8). To determine in which phase of the cell cycle cells were most responsive to induction, each of these sorted subpopulations was exposed to 2 h of darkness, and the cell cycle distributions were determined at the end of the dark interval. This short period of darkness was used as the cue to minimize the amount of cell cycle progression that occurred during the signal.

The cell cycle distributions of the populations after the dark interval were well correlated with the mean relative chlorophyll fluorescence of the sorted samples (Fig. 9A). Those populations with the lowest mean chlorophyll fluorescence represented populations with the highest proportion of  $G_1$  cells, and those populations with the highest mean chlorophyll fluorescence contained the highest proportion of  $G_2$  cells. A strong correlation between the proportion of cells in  $G_1$  after induction and the percentage of the population which formed gametes in the light was observed (Fig. 9B) indicating that cells in  $G_1$  responded preferentially to the induction cue.

## DISCUSSION

We found that the centric diatom, *T. weissflogii*, can be induced to undergo spermatogenesis in response to a decrease in photon flux density. Responsiveness to this cue is unrelated to cell size in the populations we examined. Rather, our evidence strongly suggests that cells preferentially respond to induction signals during a limited portion of their cell cycle. This dependence of developmental options on the location of a cell in its cell cycle has been observed in a number of other eukaryotes (e.g. Kates and Jones 1964, Schmeisser et al. 1973, Pringle and Hartwell 1981, Clegg et al. 1987, Gomer and Firtel 1987, Hoffmeister and Schaller 1987), and translates into an enormous amount of diversity in the behavior of diatom populations under changing light conditions.

The evidence supporting the hypothesis that the susceptibility of a *T. weissflogii* cell to an induction signal is dependent on the cell cycle has several dimensions. First, within a given population, the maximum number of cells that form gametes when returned to the light is proportional to the number of cells that divide in the dark; once cell division stops in the dark, no more cells are triggered to undergo spermatogenesis even if the duration of the dark interval is lengthened (Fig. 5). Cell cycle progression is thus implicated as a necessary component of induction. Second, the use of populations with discrete cell cycle distributions

Fig. 8. A) Distribution of chlorophyll fluorescence per cell of an exponentially growing population. The vertical lines indicate the boundaries of sorting windows. B) Distribution of chlorophyll fluorescence per cell of the sorted samples.

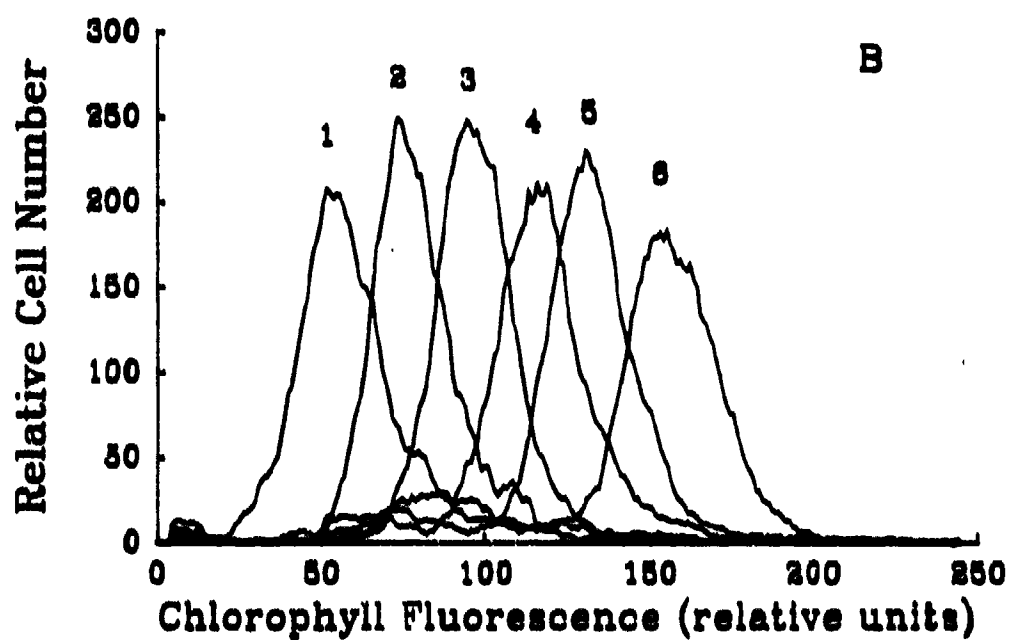
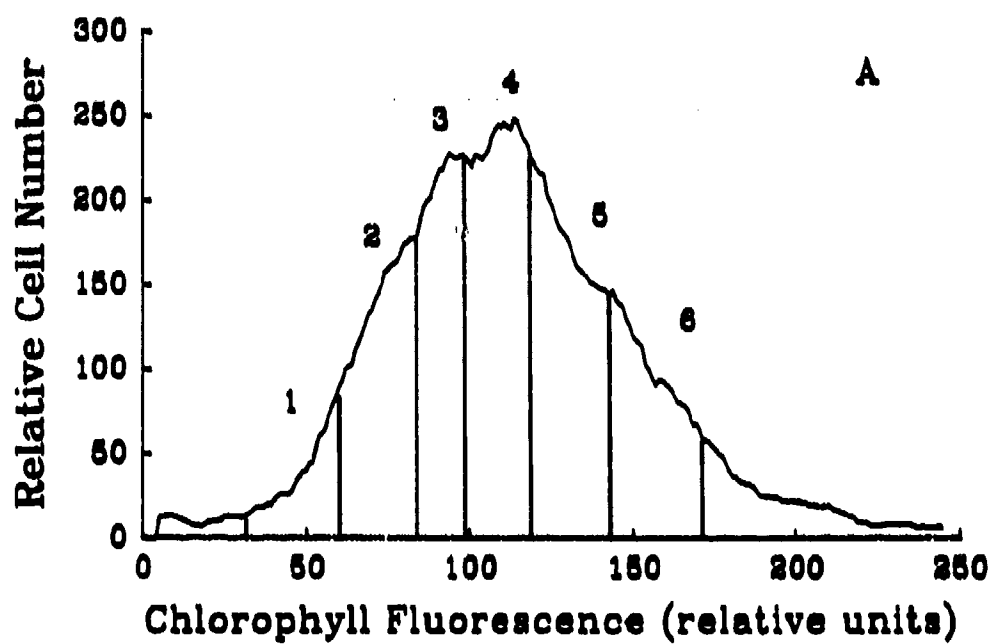
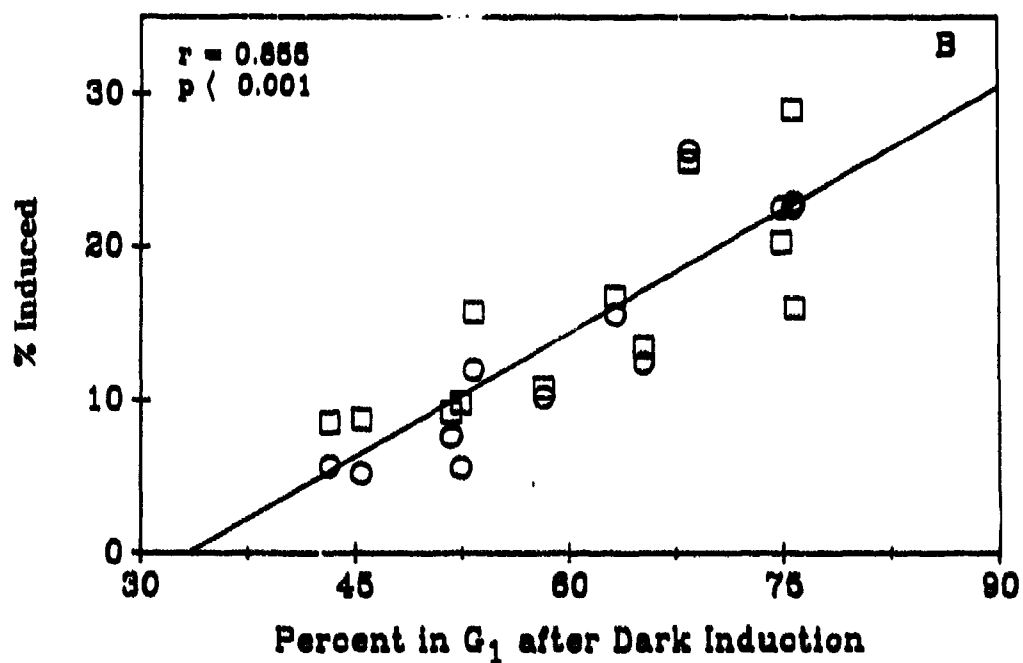
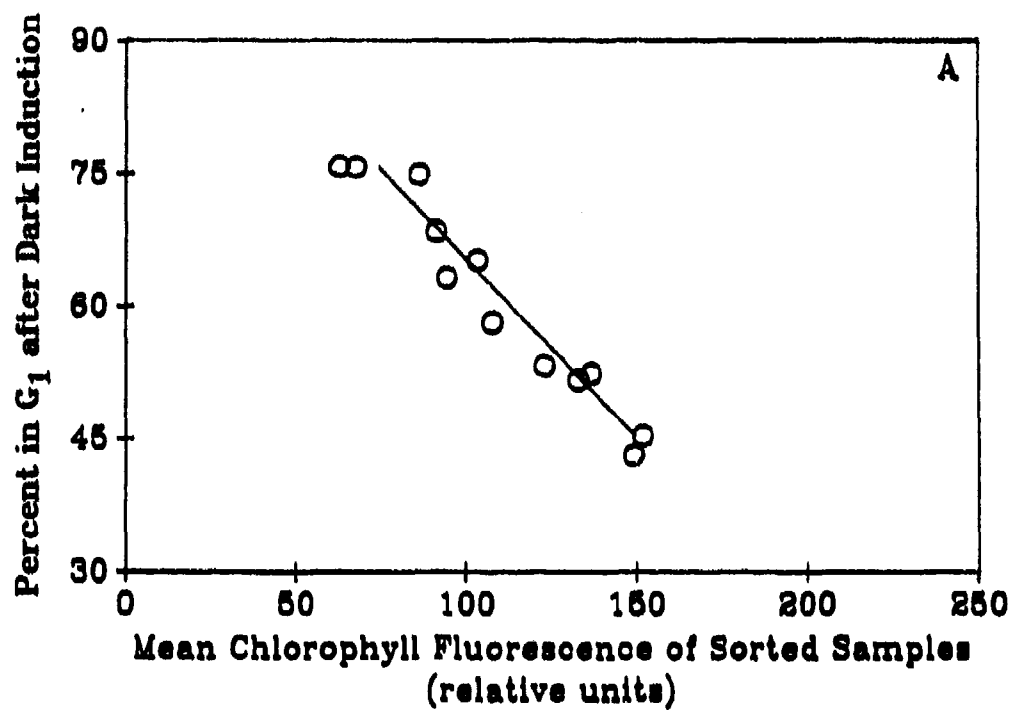


Fig. 9. A) Mean chlorophyll fluorescence of the sorted samples described in Fig. 8 versus the percentage of  $G_1$  cells in each sample after the 2 h dark induction interval. (B) Maximum percentage of each of the sorted subpopulations which underwent spermatogenesis after a 2 hour dark exposure. In (B), samples were analyzed 14 h (O) and 18 h (□) after release into continuous light.



indicates that the magnitude of a population response is directly related to the number of  $G_1$  cells present within the culture when the dark induction cue is imposed (Fig. 9B). This suggests that  $G_1$  cells are more responsive to the induction signal than cells in other stages of the cycle. Furthermore, the non-zero X axis intercept of Fig. 9B indicates that even within the  $G_1$  population, all the cells are not equally responsive to the cue. Assuming that chlorophyll fluorescence is a measure of cell age (Fig. 9A), younger  $G_1$  cells will be in those populations with the highest proportion of  $G_1$  cells whereas older  $G_1$  cells will be in those populations with the lowest proportion of these cells. This suggests that populations with a high proportion of young  $G_1$  cells are the most responsive to the induction signal. Taken together, these results indicate that, similar to the freshwater alga, *Chlamydomonas reinhardtii* (Kates and Jones 1964, Schmeisser et al. 1973), *T. weissflogii* responds preferentially to an induction trigger during an inducible region located in early  $G_1$ ; cells in the remaining portions of the cycle essentially ignore the signal and continue to divide mitotically (Fig. 10).

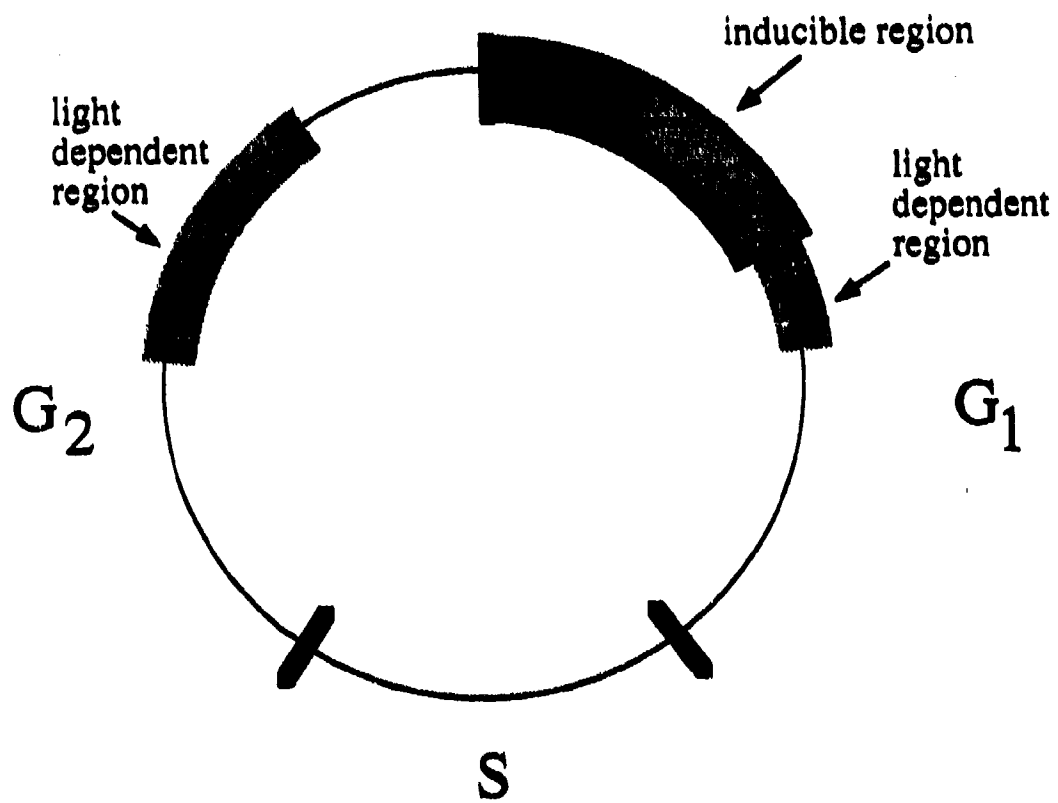
To fully explain each of the observed responses of populations to a change in light conditions in terms of this model, the manner in which light affects cell cycle progression must first be examined. The *T. weissflogii* cell cycle contains two light dependent regions, one in  $G_1$  and another in  $G_2$ , where continued cell cycle progression requires light (Vaulot et al. 1986, Olson et al.

1986) (Fig. 10). The amount of time necessary to traverse these segments increases when light is limiting. In the dark, cell cycle progression ceases completely, and cells arrest in either  $G_1$  or  $G_2$ .

With this as a framework, the behavior of populations under a range of light regimes can be interpreted. A key predictor of a population response under a given set of conditions is the number of cells which move through the inducible region. When a culture which has been maintained in exponential growth is transferred to an extended period of darkness, the total number of cells which are past the  $G_2$  but before the  $G_1$  light dependent region determines the maximum number of cells which can move into the inducible region during the dark interval (Fig. 10). The longer the population is maintained in the dark, the greater the number of cells which will move through the inducible region until eventually cell cycle progression ceases in each cell. At this point, all the induced cells will be located in the light dependent region of  $G_1$  (Fig. 10), and one can not further increase the percentage of the population induced to undergo spermatogenesis by extending the dark period (Fig. 5). Since gametic differentiation does not occur until the population is returned to the light (Fig. 1), cells must exit the mitotic cycle somewhere after this  $G_1$  dark arrest point. A burst in the initiation of spermatogenesis is thus observed as the induced cells move out of the light dependent region and exit the cell cycle (Fig. 3B).



Fig. 10. Schematic of the relationship between the mitotic cell cycle and responsiveness to an induction signal. Location of the light dependent segments of the cell cycle is based upon studies by Vaulot et al. (1986). The important features of this model are the relative positions of the various segments rather than the actual durations of each segment.



A major difference between a transfer to darkness and a transfer to subsaturating PFDs is that cell cycle progression eventually ceases in cells kept in the dark, whereas it merely slows in cells maintained in dim light. After a transfer to low light, therefore, each cell within a given population should eventually move through the inducible region; more time will be required for this to occur under more light limiting conditions. The fact that the proportion of cells that undergo gametogenesis does vary with the light intensity of the shift (Figs. 6, 7) therefore indicates that at least one other factor must limit the number of cells which are actually induced. We hypothesize that the apparent "decay" of the cue (Fig. 6) results from the fact that phytoplankton are able to photoadapt to new light conditions (e.g. Falkowski 1980); eventually the shift to low light is no longer sensed as a new light level, and all the cells divide mitotically. The maximum percentage of cells induced to form gametes under subsaturating light will thus be determined by the duration of the cell cycle in the new light conditions relative to the length of time that low light functions as a trigger.

The induction of spermatogenesis in *T. weissflogii* is undoubtedly more complex than has been described thus far. For example, the slope of Figure 5C indicates that approximately 60% of the cells which divided during the dark interval and thus moved into the inducible region actually formed gametes in the light. Also, the scatter observed in Figure 4A cannot be explained even if the

responses of all our various isolates are plotted against a measure of the number of cells which move into the inducible region (the number of cells which divided during the dark interval). Although a statistically significant positive correlation is obtained ( $r=0.549$ ,  $P<.001$ , data not shown), a great deal of the variability remains unexplained. This cell cycle model can thus predict the responses within a given population, but it is inadequate for predicting the responses between different populations.

Induction of spermatogenesis in *T. weissflogii* may in fact be a two step process similar to the induction of meiosis in the budding yeast, *Saccharomyces cerevisiae* (Mitchell 1988). In yeast, nutrient limitation fulfills the first requirement for induction, but only those cells which are heterozygous at the mating locus are actually able to undergo meiosis (Herskowitz 1988). Perhaps movement of cells through the inducible region of  $G_1$  in dim light or darkness fulfills the first requirement in diatoms, but as with yeast, the cell must also possess a particular genetic makeup in order to fulfill the second requirement. If this is indeed the case, it may explain the wide range of responses we observed among our various cultures; our method of obtaining the original isolates undoubtedly resulted in genetic differences among these populations. Thus, certain populations may be particularly susceptible to an induction cue whereas others may be relatively unresponsive. Moreover, although diatoms are believed to be monoecious (i.e. both males and females can result from a single clone), we have yet to

observe any evidence of the presence of females in our induced cultures. Perhaps these unresponsive or "sterile males" are actually females awaiting a proper induction signal.

Predictions of the number of cells that will undergo spermatogenesis in response to an induction signal thus require that at least three layers of complexity be taken into consideration: first, the number of cells which move through the inducible region; second, the amount of time that a shift in PFD functions as an induction signal; and third, the number of cells which may be functionally sterile in their response to a change in light conditions.

What are the implications of these results for diatom populations in the field which are exposed to a decrease in PFDs on a daily basis? The maximum extent of gametogenesis in a population depends not only on the presence of an induction signal but also upon the manner in which this signal influences cell cycle progression. If the cell cycles of a population of cells are aligned such that few cells move through the inducible region during the night, for instance, then a minimum number of cells will be induced to form gametes. In this regard, it is interesting to note that in contrast to other eukaryotic phytoplankton, diatoms divide predominantly during the light interval (e.g. Nelson and Brand 1979, Chisholm et al. 1984). Moreover, Chisholm and Costello (1980) found that in *T. weissflogii*, the timing of the division burst (i.e. an approximation of the interval when cells should move through the

inducible region) on a light/dark cycle varies with temperature, the duration of the light interval, nutrient status, and even cell size. Thus, realistic predictions of the extent of sexuality that occurs in the field require a greater understanding of those factors that function as induction signals and the manner in which these factors influence cell cycle progression.

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CHAPTER 3

PATTERNS OF CELL SIZE CHANGE IN A MARINE CENTRIC DIATOM



### Abstract

During nearly two years of exponential growth, isolates of the centric diatom, *Thalassiosira weissflogii*, displayed periodic increases and decreases in mean cell size as predicted for diatom populations alternating between asexual and sexual reproduction. However, despite the fact that each culture was maintained in constant, identical conditions, the overall patterns of cell size changes displayed by the various populations were unique, complex and unpredictable. The onset of sexual reproduction, the rate of increase in average cell size, and the size of the post-auxospore cells created during a sexual event varied among isolates and even within a given isolate over time; the only constant feature among the various cultures was the rate of decrease in mean cell size. We hypothesize that the extent of the variability exhibited by the various populations results ultimately from the fact that the genetic composition of diatom populations varies over time.

## Introduction

Beginning as early as the mid 1800's, studies have indicated that the diatom life cycle is influenced by the unique configuration of its cell wall, a frustule composed of two unequally sized silica valves encircled by a series of siliceous girdle bands (e.g. MacDonald 1869, Geitler 1935, von Stosch 1965, Drebes 1977). Due to the physical constraints of the frustule, each mitotic division creates one daughter cell whose diameter is smaller than that of her sister or her mother by twice the thickness of these girdle bands. For most diatoms, therefore, the mean cell size of a population decreases and the standard deviation about this mean increases over successive generations. Perhaps not surprisingly, though a number of exceptions to this general rule of size reduction have been observed (e.g. Rao and Desikachary 1970, Round 1972, Crawford 1980).

The most common manner of escaping the trend of diminishing cell size is through sexual reproduction (Drebes 1977). In response to a range of environmental cues including sudden changes in light, temperature, salinity or nutrient conditions (e.g. Steele 1965, Drebes 1966, Schultz and Trainor 1968, Furnas 1985, French and Hargraves 1985, Vaulot and Chisholm 1987), diatoms can become sexual; meiosis replaces mitosis and the resulting male and female gametes fuse to form a zygote or auxospore. This auxospore escapes its frustule to create a post-auxospore cell many times larger than either parent (e.g. Findlay 1969). In addition to their new large size, post-auxospore cells apparently also possess an increased

"vigor" since they are often observed to grow at faster rates than those of the relatively small cells from which they were created (Paasche 1973, Costello and Chisholm 1981). An alternative, but rarely observed means of restoring cell size occurs asexually, generally in response to the alleviation of extreme nutrient deprivation. In this circumstance, a vegetative cell simply extrudes its cytoplasm into the surrounding waters and develops a new and much larger frustule (e.g. von Stosch 1965, Drebes 1966, Gallagher 1983, French and Hargraves 1986).

Whether created asexually or sexually, large cells are considered to be incapable of responding to sexual induction signals for several generations, until a permissive size range, approximately 30-40 percent of a species' maximum diameter, is obtained (Drebes 1977). Within this permissive range, an even further refinement of the size control of sexuality has been observed; often, smaller cells appear to preferentially undergo spermatogenesis while larger cells tend towards oogenesis (e.g. Geitler 1952, Migita 1967, Rao 1971, French and Hargraves 1985). Moreover, the signals inducing the cells to undergo either spermatogenesis or oogenesis may differ slightly (Holmes 1966, Drebes 1977). Thus, within an actively growing diatom population there exists an assortment of cell sizes with distinctly different physiologies (e.g. Werner 1971) which makes the study of diatom life cycles extremely complex.



We recently examined whether the putative size requirement for sexual differentiation could account for the proportion of a population that was triggered to undergo spermatogenesis in response to a change in light conditions (Armbrust et al. 1990). To our surprise, we found that for a number of isolates representing a broad range of size distributions, there was no correlation between average cell size and the proportion of a population that could be induced to undergo spermatogenesis. While attempting to interpret these data, we found that no long term studies existed which described in detail the evolution of cell size in an exponentially growing diatom populations. Although there are many predictions for the change in cell size over time, no data exists on the development of these changes through more than one round of sexuality (e.g. Findlay 1969, Rao 1971). Thus, we initiated a long-term study to document exactly how, under optimum growth conditions and in the absence of external signals, cell size changes in diatom populations over successive generations.

#### Materials and Methods

Isolates of *T. weissflogii* Grun. clone Actin (from the Culture Collection of Marine Phytoplankton, Bigelow Laboratories for Ocean Sciences) were obtained by isolating colonies from 2% agar plates and transferring them to f/2 enriched seawater media (Guillard 1975) with nitrate as the nitrogen source. Each isolate was maintained in semi-continuous batch cultures at 20°C and saturating levels of

continuous illumination ( $250 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$ , cool-white fluorescent) (Armbrust et al. 1990). After approximately 120 days in exponential growth, new populations were initiated by plating the parent culture and isolating additional colonies (Fig. 1). Each isolate was then split into replicates; at least 10,000 cells were used for each innoculum. Throughout the following discussion, the term isolate rather than clone will be used. Although each population arose from a single cell, diatoms are diploid and genetic recombination during sexual reproduction prevents cultures from remaining genetically homogeneous and thus clonal over time.

In an attempt to obtain genetically identical populations, individual cells from known cell lineages were isolated and cloned. These single cell isolations were obtained by viewing an aliquot of two different cultures through a dissecting microscope and manually picking individual cells from the liquid media. Each of four isolated cells (two from each culture) was transferred to its own well (within a 96-well plate) containing f/2 media. Approximately 24 hours later, each daughter cell was removed from a given well, transferred to its own well, and allowed to develop into a population. In this manner, cultures originating from 15 single cell isolations of known relationships were obtained (Fig. 2). These cultures were also maintained in semi-continuous batch culture at  $20^{\circ}\text{C}$  and  $250 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$  of continuous light. Cultures descended from the same mother cell were considered to be genetically identical until the onset of sexual reproduction.

Figure 1. Cell lineages and initial Coulter volume distributions of *T. weissflogii* populations. Each isolate is designated by a number corresponding to its mean Coulter volume ~2 weeks after transfer from colonies on agar plates to liquid medium. The laboratory stock culture was plated and two isolates, 868 and 1724 were isolated and maintained in exponential growth. An aliquot of 868 was plated and two new isolates, 709 and 877 were initiated. In the course of conducting experiments with isolate 868, the average Coulter volume of one experimental flask spontaneously increased suggesting that sexual reproduction had occurred. This newly enlarged population was designated 1147. After approximately 120 days of exponential growth, aliquots of 1724, 877, and 1147 were plated and 6 new isolates, 637, 834, 485, 747, 444, and 920 were initiated. Once each isolate was transferred to liquid media, the population was maintained in exponential growth. In each Coulter volume distribution panel, "initial" refers to the Coulter volume distribution of a population soon after isolation and "subclnd" refers to the Coulter volume distribution of the same population at the time the derivative isolations were made.

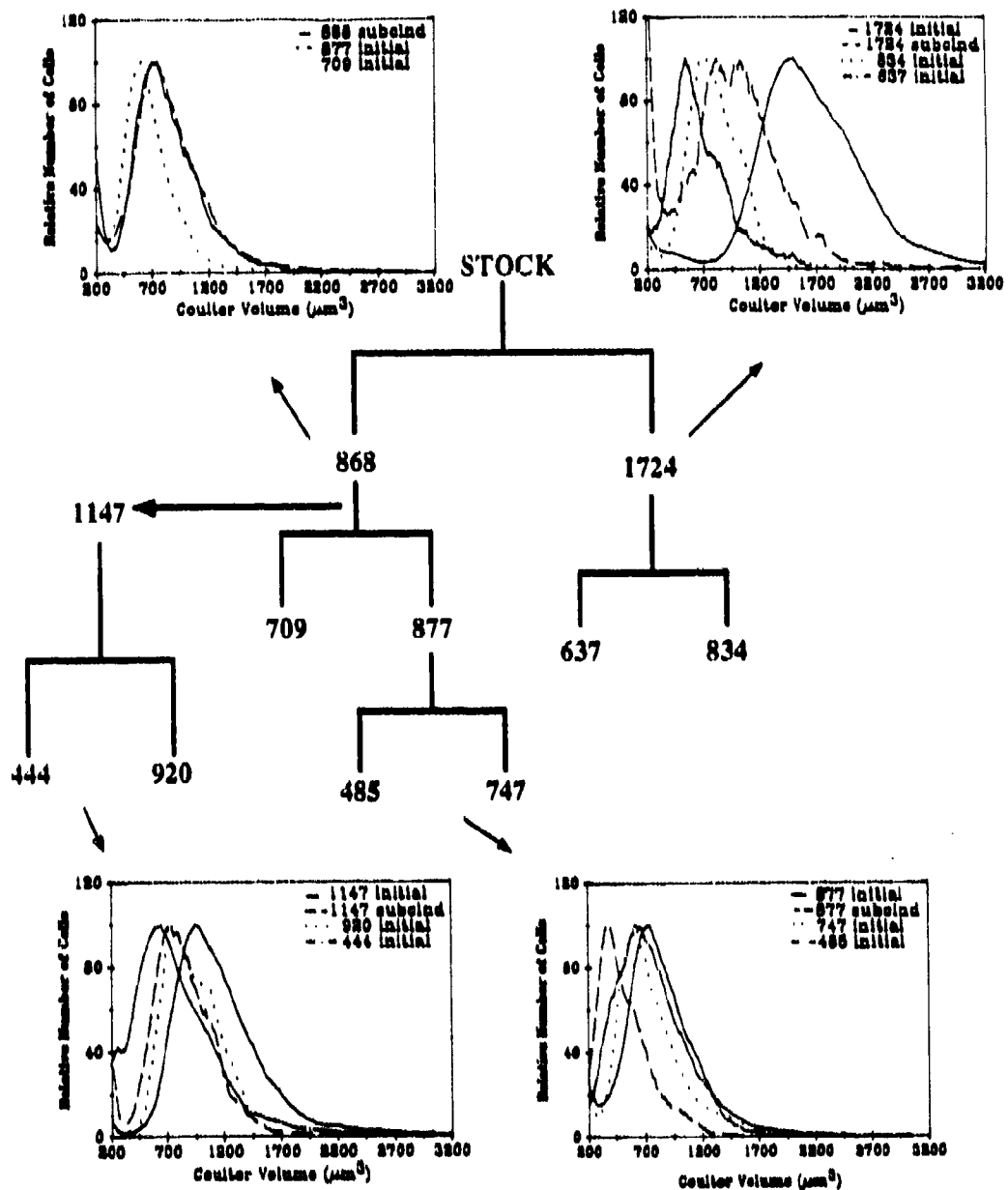
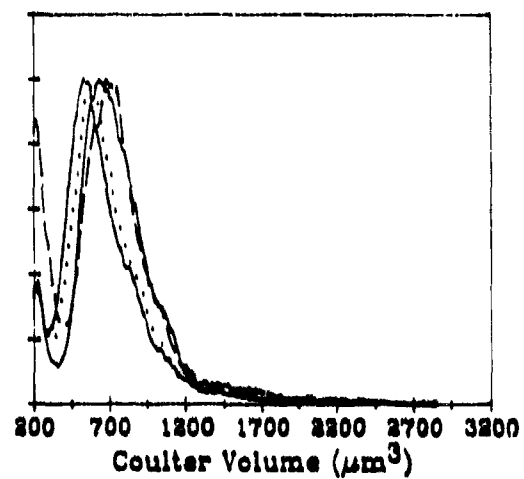
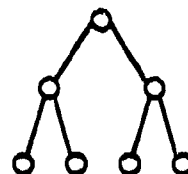
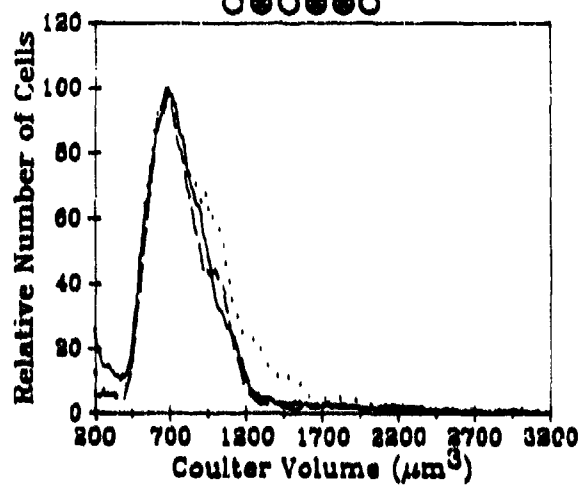
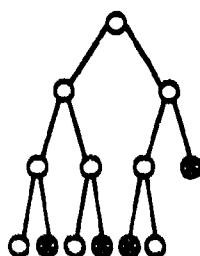
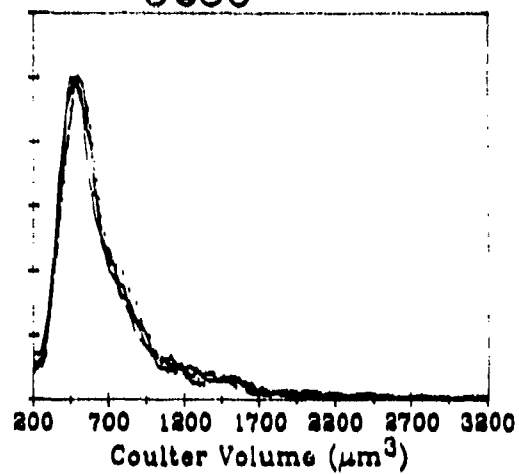
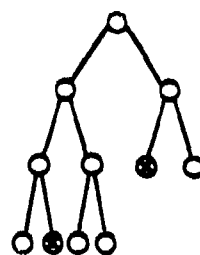
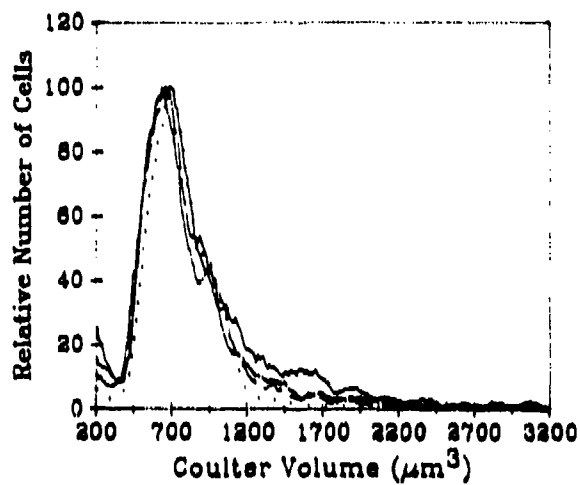
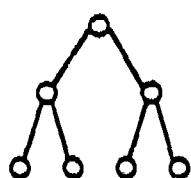


Fig. 2. Cell lineage and initial Coulter volume distributions of populations derived from genetically identical cells (A, B, C, D). The four original cells were isolated from replicate cultures of isolate 1724 (1724-1 & 1724-2). The number of branches in a family tree is based on the number of cells observed in each well at the end of 24 hours and indicates the hypothesized number of divisions that occurred before the cells were reisolated. The X in certain circles indicate that some of the reisolated cells did not develop into cultures. The Coulter volume distributions of each population approximately 2-3 weeks after the initial isolation are shown below each tree. The different line types indicate the individual cell lines.



Size distributions and the mean cell size of each population were determined with a Coulter Electronics Model Z<sub>m</sub>/C256 electronic particle counter. The within-measurement error was estimated by determining the Coulter volume distributions of multiple aliquots of a given population; the mean Coulter volumes of these subsamples varied by less than 5 %. The between-measurement error over consecutive days was estimated by determining the extent of the scatter during a linearly decreasing interval of mean Coulter volume. Although random outliers were seen, the greatest persistent scatter observed was approximately 40%; the vast majority of cultures, however, displayed substantially less spread, in many instances an amount comparable to the within measurement error. The coefficient of variation (CV = standard deviation/mean) was determined for each size distribution.

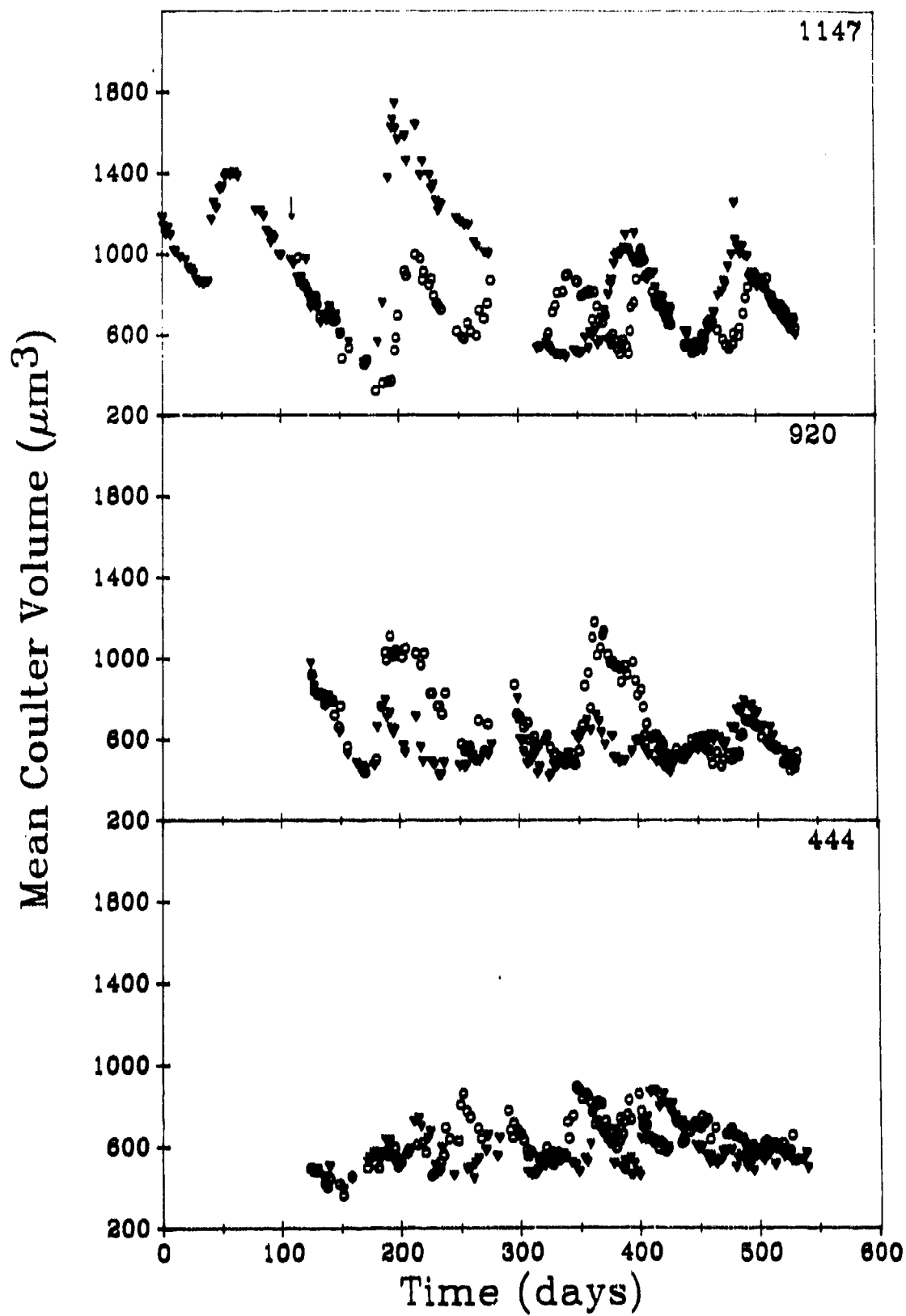
A Turner fluorometer was used to monitor daily changes in the *in vivo* fluorescence of each culture to determine growth rates (Brand et al. 1981). An inoculum of at least 10,000 cells were transferred to approximately 30 ml fresh media while the *in vivo* fluorescence was still less than about twenty percent of the maximum fluorescence. Thus, new cultures were inoculated every 2-3 days. Before each transfer, the Coulter volume distribution was determined.

## Results

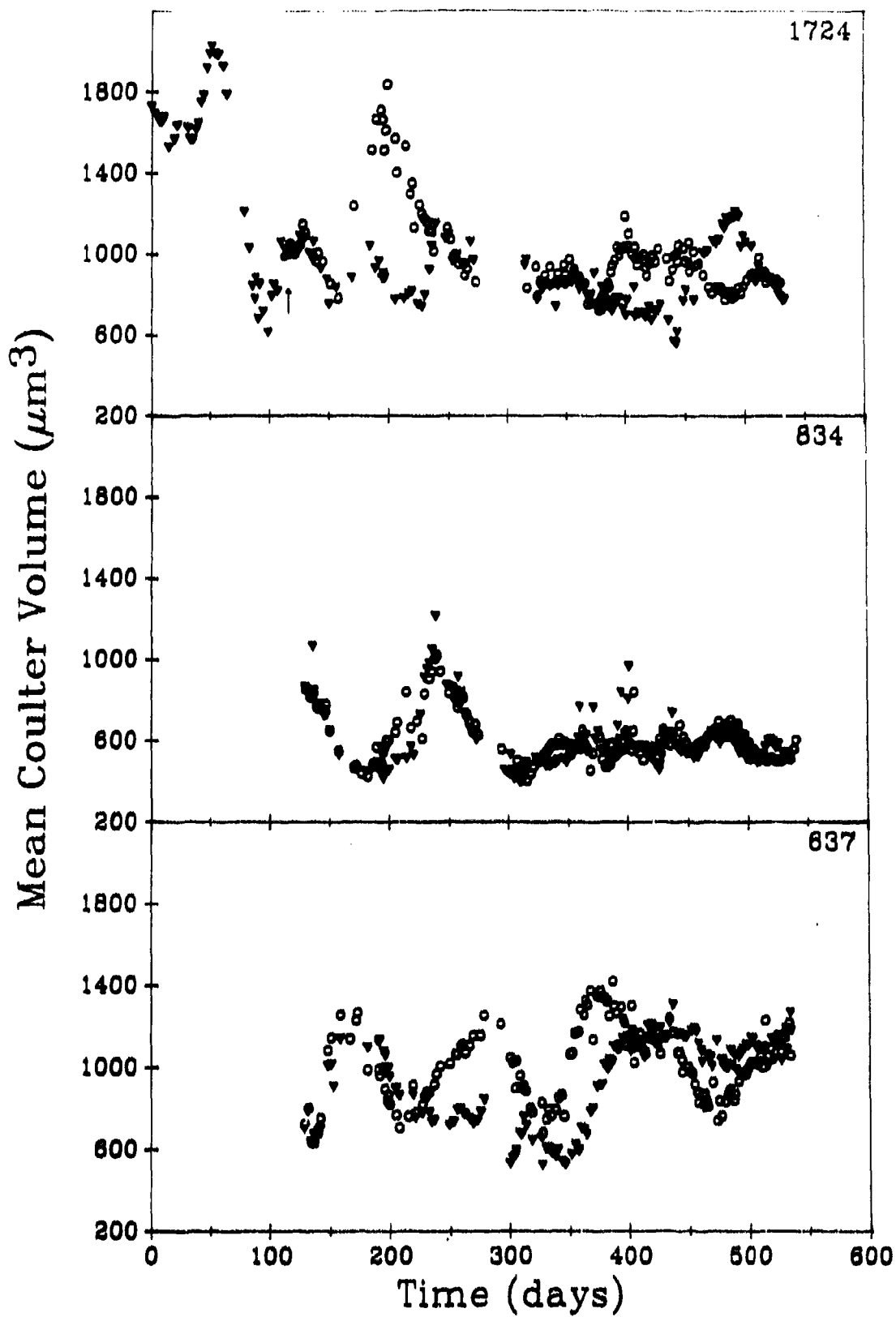
*Evolution of mean Coulter volume over time.* Coulter volume distributions of a series of isolates representing a broad range of cell sizes and cell lineages (Fig. 1) were monitored for nearly two years to determine the long term trajectories of cell size in *T. weissflogii* populations. Each isolate displayed periodic increases and decreases in mean cell size. However, the overall patterns of the cell size changes displayed by each culture varied dramatically despite the fact that each population was maintained under identical conditions. The timing, rate of increase, and amplitude of the size oscillations differed between isolates, within a given isolate over time, and even between replicate flasks of a single isolate (Figs. 3-6). For instance, a number of the populations, regardless of their size distribution upon isolation (see Fig. 1), underwent dramatic increases in average cell size early on, only to later undergo smaller, more rapidly occurring oscillations (Figs. 3A, 4A & B, 5B & C). Other isolates maintained a relatively small size throughout the study although increases and decreases in size were still apparent (Figs. 3C, 5A, 6A, B & C). Moreover, the rate of increase in average Coulter volume in the various isolates ranged from a maximum of nearly  $23 \mu\text{m}^3 \cdot \text{cell}^{-1} \cdot \text{gen}^{-1}$  (Fig. 3A) to a minimum of about  $1.2 \mu\text{m}^3 \cdot \text{cell}^{-1} \cdot \text{gen}^{-1}$  (Fig. 4B).



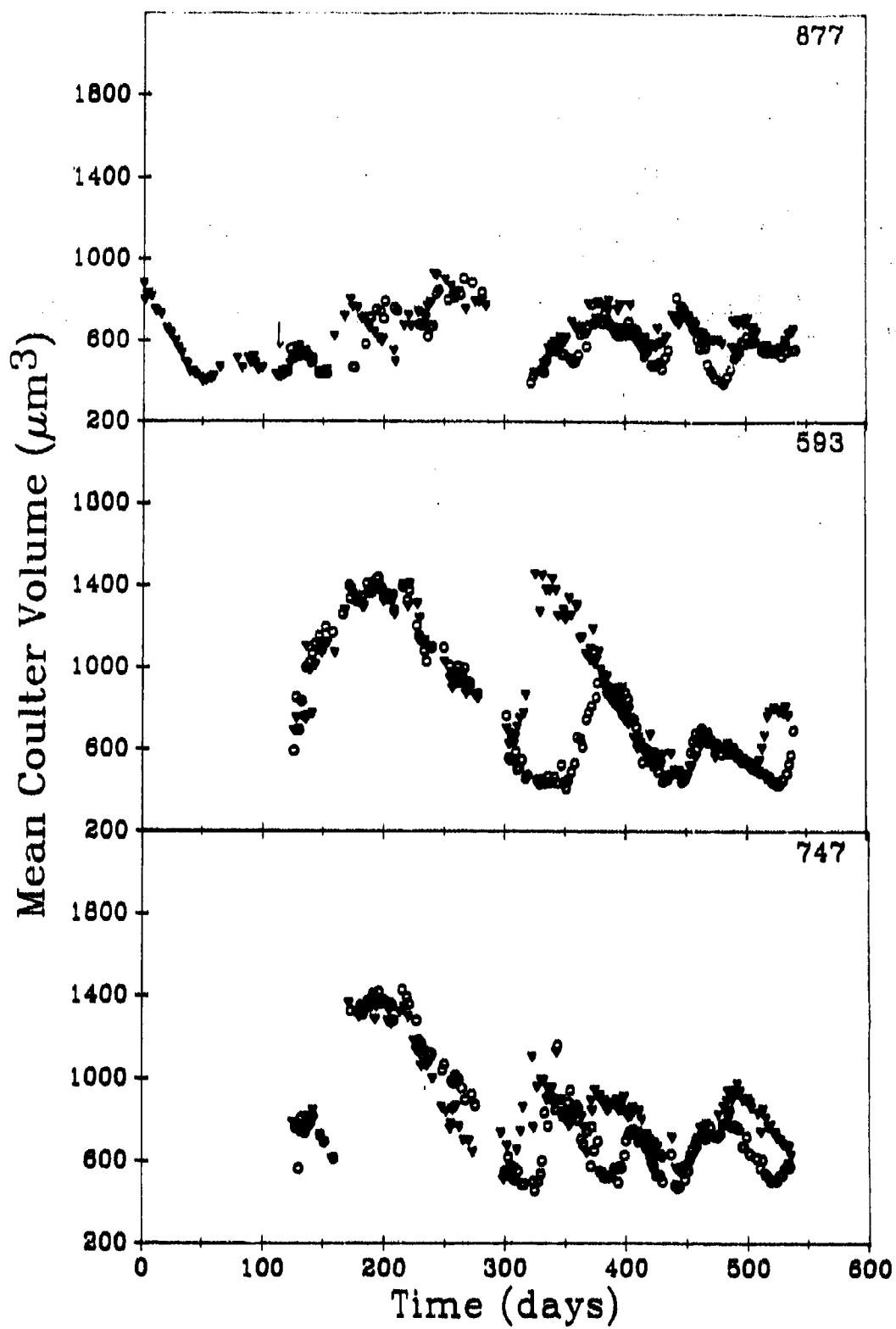
Figs. 3. Mean Coulter volume over time of the *T. weissflogii* isolates presented in Fig. 1. A) Population 1147, was maintained in exponential growth and beginning at t=0 days, the Coulter volume distributions were monitored over time. At t=127 days, the culture was split into replicate flasks (darkened triangles and open circles). B, C) At t=120 days (as indicated by arrow in (A)), 1147 was plated and two new isolates, 920 and 444, were initiated. At t=127 days, these new cultures were split into replicate flasks (darkened triangles and open circles) and the Coulter volume distributions of each were monitored over time.



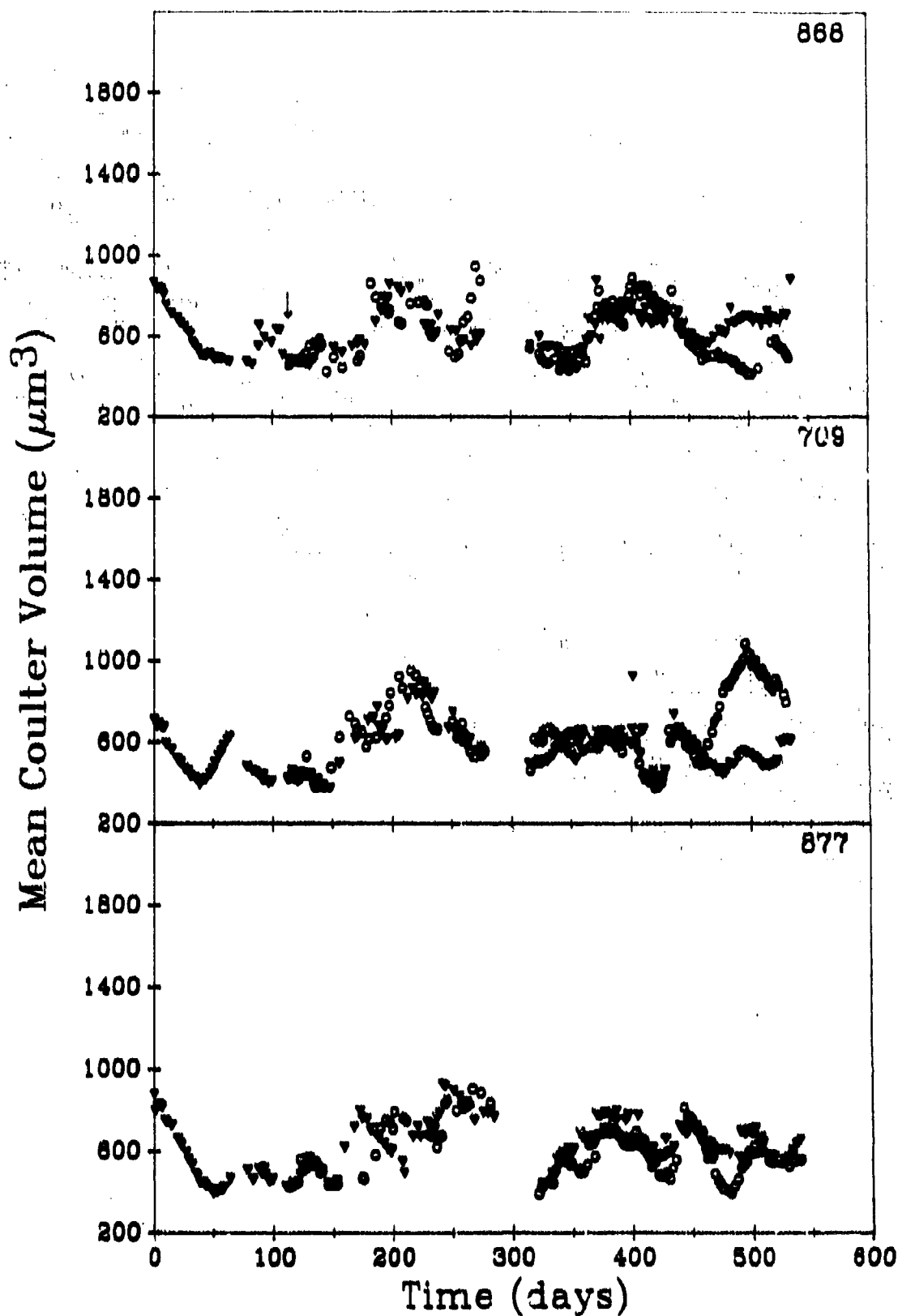
Figs. 4. Mean Coulter volume over time of the *T. weissflogii* isolates presented in Fig. 1. A) Isolate 1724 was maintained in exponential growth and beginning at t=0 days, the Coulter volume distributions were monitored over time. At t=127 days, the culture was split into replicate flasks (darkened triangles and open circles). B, C) At t=120 days (as indicated by arrow in (A)), 1724 was plated and two new isolates, 834 and 637, were initiated. At t=127 days, these new cultures were split into replicate flasks (darkened triangles and open circles) and the Coulter volume distributions of each were monitored over time.



Figs. 5. Mean Coulter volume over time of the *T. weissflogii* isolates presented in Fig. 1. A) Isolate 877 was maintained in exponential growth and beginning at t=0 days, the Coulter volume distributions were monitored over time. At t=127 days, the culture was split into replicate flasks (darkened triangles and open circles). B, C) At t=120 days (as indicated by arrow in (A)), 877 was plated and two isolates, 593 and 747, were initiated. At t=127 days, these new cultures were split into replicate flasks (darkened triangles and open circles) and the Coulter volume distributions of each were monitored over time.



Figs. 6. Mean Coulter volume over time of the *T. weissflogii* isolates presented in Fig. 1. A) Isolate 868 was maintained in exponential growth and beginning at t=0 days, the Coulter volume distributions were monitored over time. At t=127 days, the culture was split into replicate flasks (darkened triangles and open circles). B, C) At t=120 days (as indicated by arrow in (A)), 868 was plated and two new isolates, 709 and 877, were initiated. At t=127 days, these new cultures were split into replicate flasks (darkened triangles and open circles) and the Coulter volume distributions of each were monitored over time.





Perhaps the most surprising result of all was that replicate cultures did not always behave as replicates; they consistently "tracked" each other only during the initial downward swing in average Coulter volume (during this interval, the slopes and elevations of the replicate lines were not significantly different,  $p > 0.5$ , Student's *t* test). Replicates sometimes diverged when one of the cultures initiated cell enlargement (for example, Figs. 3A & B, 5B & C); the onset of this size increase might (e.g. Figs. 3B, 4) or might not (e.g. Fig. 3A) occur simultaneously in a pair of replicates.

Since diatoms are known to decrease in cell size during mitotic divisions, the intervals of decreasing mean Coulter volume were assumed to reflect a predominance of asexual reproduction. The intervals of increasing mean Coulter volume were assumed to reflect the formation of auxospores, a process that can occur both sexually and asexually. Because none of the cultures experienced nutrient deprivation, asexual cell enlargement (Drebes 1966, Gallagher 1983) was considered an unlikely source of the observed increase in cell size. Moreover, von Stosch (1965) was unable to induce asexual cell enlargement in any of his *Thalassiosira* species, so this type of auxospore formation may not be possible in this genus. Therefore, for the purposes of our discussion, the intervals of increasing mean Coulter volume are assumed to reflect the sexual formation of auxospores.

The onset of sexual reproduction in each of the various cultures was staggered in time in an unpredictable manner (Fig. 3-6) thus eliminating the possibility that an unintentional and undetected fluctuation in the environment (such as the quality of different batches of seawater) triggered the initiation of these sexual events. Some isolates underwent sexual reproduction and auxospore formation as frequently as every 120 generations (about 2 months, e.g. Fig. 3A) while other populations remained asexual for up to 450 generations (approximately 8-9 months, e.g. Fig. 5B).

The extent of variation observed in the behavior of the various populations despite constant growth conditions, indicated underlying genetic variability among the isolates. Cell lineage (Fig. 1), however, predicted population behavior only slightly better than did the initial size distributions. This is not surprising since each of the original mother isolates had undergone at least one round of auxospore formation and thus sexual reproduction by the time the new daughter isolates were initiated (Figs. 3A, 4A, 5A, 6A). Genetic recombination in these diploid cells during sexual reproduction undoubtedly created the variability in behavior. For example, the average cell size of two isolates, 877 and 868, remained relatively small throughout the study (Figs. 5A, 6A), but only in the 868 lineage did the descendants also remain small (Fig. 6). Descendants of isolate 877 underwent relatively large size increases (Fig. 5). Similarly, the behavior of population 1147, which presumably arose from 868 via sexual

reproduction (Fig. 1), not only contrasted sharply with that of the population from which it was derived, but also with that of its own descendents (Fig. 3). And finally, isolate 1724 which was engaged in auxospore formation when the new isolates were initiated, yielded two populations (834 and 637) one of which was on the downswing of the cycle (Fig. 4B) and the other of which was on the upswing (Fig. 4C), suggesting that the progenitor of 834 had recently undergone sexual reproduction while the progenitor of 637 was still a few generations away.

Against this backdrop of variability, the rate of decrease in mean cell size was relatively constant; on average, cells decreased in Coulter volume by about  $8 \mu\text{m}^3 \cdot \text{cell}^{-1} \cdot \text{gen}^{-1}$  (Table 1). The expected rate of decrease in volume during asexual reproduction can be calculated based on an estimate of the thickness of the girdle bands attached to each frustule half. Li and Volcani (1985) have estimated that the girdle bands of the centric diatom *Ditylum brightwellii*, range from approximately 7 nm to perhaps as much as 15 nm thick (comparable to the thickness of the frustule itself). Assuming that upon mitotic division, the diameter of one daughter cell decreases by twice the thickness of these girdle bands, the volume changes in the *T. weissflogii* cultures should have varied from approximately 2 -  $11.5 \mu\text{m}^3$  per cell per generation (depending on the initial volume used in the calculations). These predictions agree remarkably well with the measured values (Table 1).

The minimum average Coulter volume achieved within a given isolate was generally constant (Figs. 3-6), as would be expected if sexual reproduction was restricted to cells within a permissive size range (e.g. Drebes 1977), but, it should be noted that in one isolate, an upswing was initiated at about  $850 \mu\text{m}^3$  (Fig. 3A) while in another isolate the upswing began at about  $1550 \mu\text{m}^3$ . This latter value corresponded to a diameter 78 % of the eventual maximum diameter (Fig. 4A) and was far in excess of the 30-40 % range generally used to define the potential onset of sexuality (Drebes 1977). Moreover, between isolates, the minimum volume ranged from 400 to  $700 \mu\text{m}^3$ . Contrary to other reports (e.g. Migita 1967, Rao 1971), this minimum average cell size did not consistently predict the maximum size obtained once an upswing was completed. Instead, the maximum average size varied greatly and populations underwent anywhere from a 1.2 to a 3.5 fold increase in mean cell size (Table 1). These results implied that at least one of two parameters could differ among populations: either the proportion of cells involved in the formation of auxospores or the maximum size obtained by any given cell.

*Coulter volume distributions.* The coefficient of variation (CV), an estimate of the extent of dispersion around a mean Coulter volume measurement, was monitored in each culture over time to determine the amount of variability in the size of the individual cells within a population and to estimate whether or not distinct subpopulations remained in a culture once the maximum average

volume was obtained. As each population decreased in average Coulter volume, the spread around the mean increased as would be predicted for populations undergoing asexual reproduction (Rao and Desikachary 1970). As each population began to undergo sexual reproduction, however, the CV decreased such that once the maximum average cell size was obtained, the variation around this new mean was at a minimum (Fig. 7). The minimum CV could vary from 25 to 45 % regardless of either the magnitude of the size increase or the maximum size obtained by the population. However, the spread around the mean volume after a sexual episode was always less than that before the cells initiated sexual reproduction suggesting that by the end of a sexual event a majority of the cells had formed comparably sized post-auxospores.

To determine whether all the cells within a culture were in fact behaving in concert as the CV data suggested, the Coulter volume distributions themselves were examined. During an interval of decreasing mean Coulter volume, populations displayed unimodal size distributions, the CV's of which gradually increased over time (Fig. 8). During the intervals of increasing mean Coulter volume, the size distribution generally developed into two distinct peaks. Over the course of anywhere from 2 weeks to a month, this bimodal distribution eventually resolved into a new unimodal distribution with a larger mean and a decreased CV (Fig. 9). The maximum size obtained by a cell was not constant and could vary between isolates (Fig. 9), within a single isolate over time (Fig. 9A, C, D), and

even between replicate cultures (data not shown). Therefore, cultures that displayed minor size fluctuations (Figs. 3C, 5A, 6) simply produced smaller post-auxospores than cultures that underwent dramatic oscillations in mean cell size (Figs. 3A, 4A & B, 5B & C).

There was no indication that distinct subpopulations with different size distributions persisted after the maximum average cell size was obtained confirming that all the cells present within the population had undergone cell enlargement by the end of a sexual event. However, it should be noted that it is impossible to determine based on size distributions alone, whether each cell within a population eventually underwent sexual reproduction or only a portion of the cells formed auxospores, the descendants of which then came to dominate the population due to an increased growth rate.

*Single cell isolations.* An analysis of the possible sources of the variability observed among our various isolates was confounded by the fact that each of the original isolates had undergone sexual reproduction prior to the initiation of replicates and additional isolates (Figs. 3-6). In an attempt to eliminate this complication, a series of cultures derived from genetically identical cousin cells were initiated (Fig. 2) and maintained under the same constant conditions as described previously. The majority of these single cell isolates underwent relatively minor size fluctuations over time (Figs. 10, 11), a behavior that essentially mimicked the minimal size changes observed in the two cultures from

Fig. 7. Mean Coulter volume (darkened triangles) and the coefficient of variation of each volume measurement (open circles) over time for a representative *T. weissflogii* population, 1147. The CV's were smoothed over three measurements.

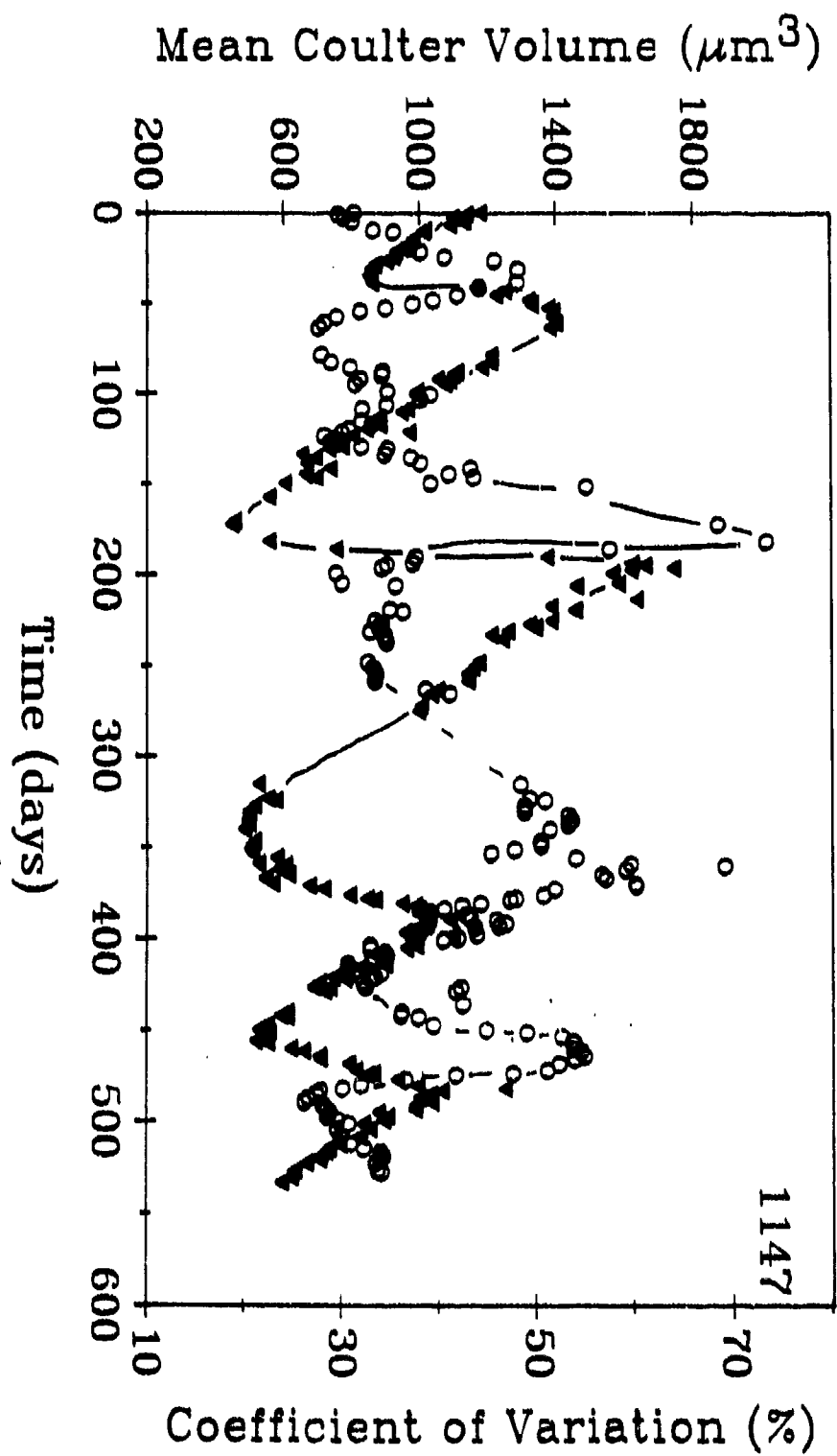




Figure 8. A) Mean Coulter volume over time of a representative *T. weissflogii* culture, isolate 747 and B) the Coulter volume distributions during the interval of decreasing mean cell size highlighted in A.

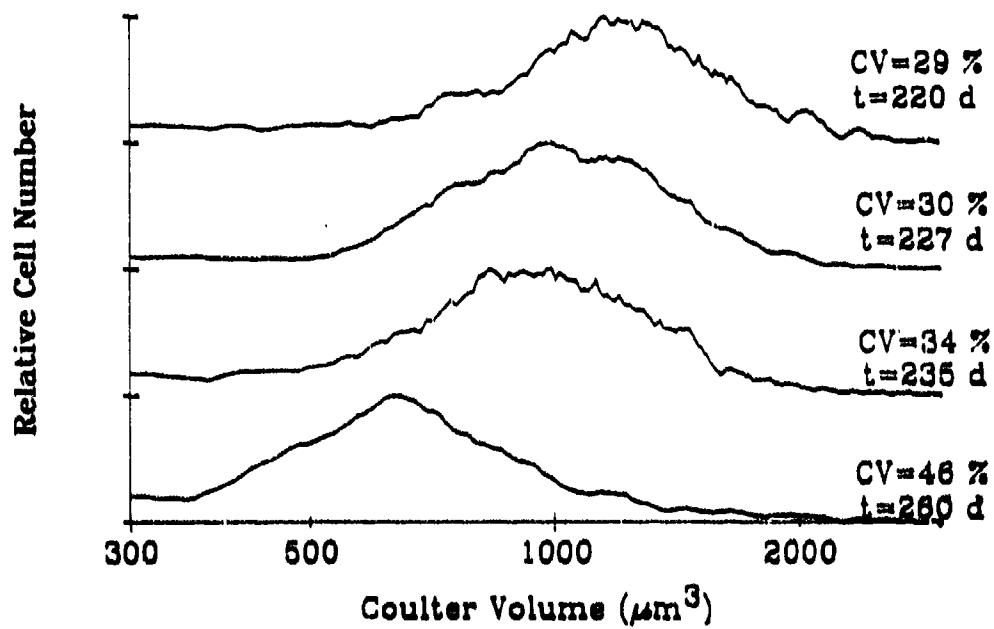
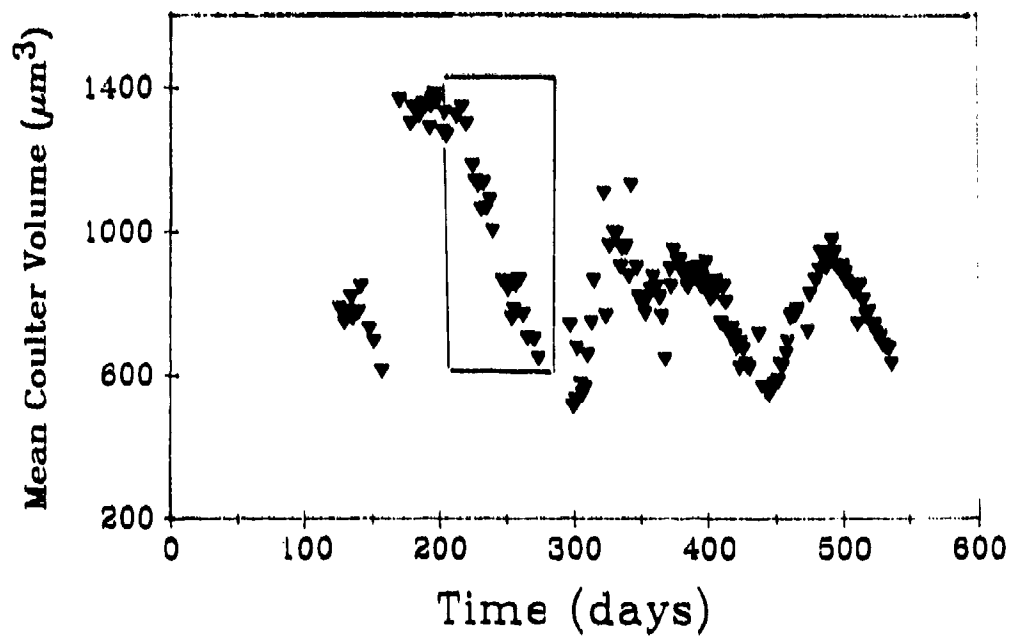


Figure 9. Mean Coulter volume over time of two *T. weissflogii* cultures and their Coulter volume distributions during the intervals of population size increase. A) Mean Coulter volume over time in a population that initially underwent a dramatic, 3.5 fold, increase in average cell size only to later undergo a series of 1.5 fold increases. B) Mean Coulter volume of a population that remained relatively small throughout the study. C, D) Coulter volume distributions over time of intervals indicated by the highlighted regions in A. E) Coulter volume distributions of interval indicated by the highlighted regions in B.

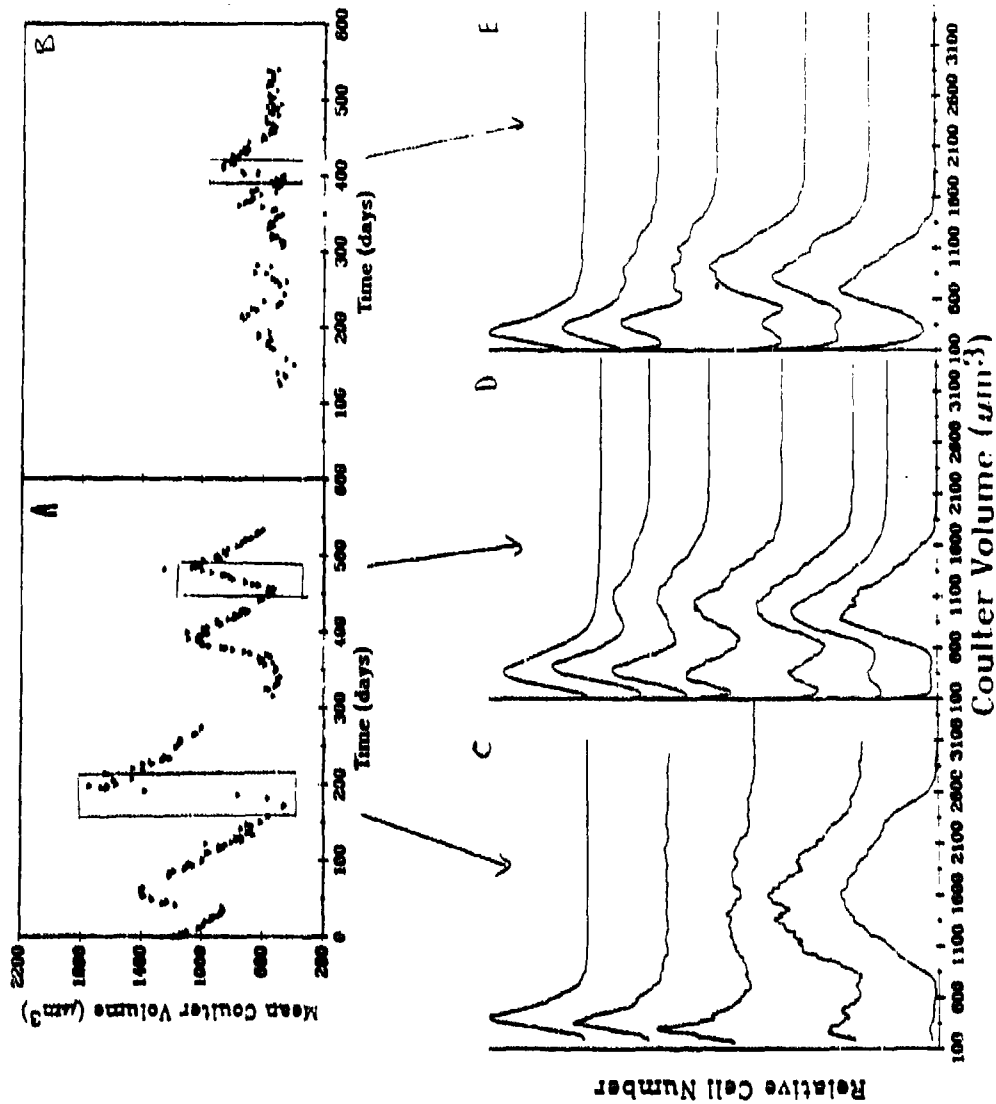


Fig. 10. Mean Coulter volume over time for two sets of populations derived from genetically identical cells of *T. weissflogii* isolate 1724-1 at t=340 days (see Fig. 4A). Each panel represents populations derived from the same grandmother or great-grandmother cell as indicated in Fig. 2. The different symbols represent the four cell lines derived from the original genetically identical cells. The insets are a blow up of the mean Coulter volume (note change in scale) during the first 20 days after the initial isolation.

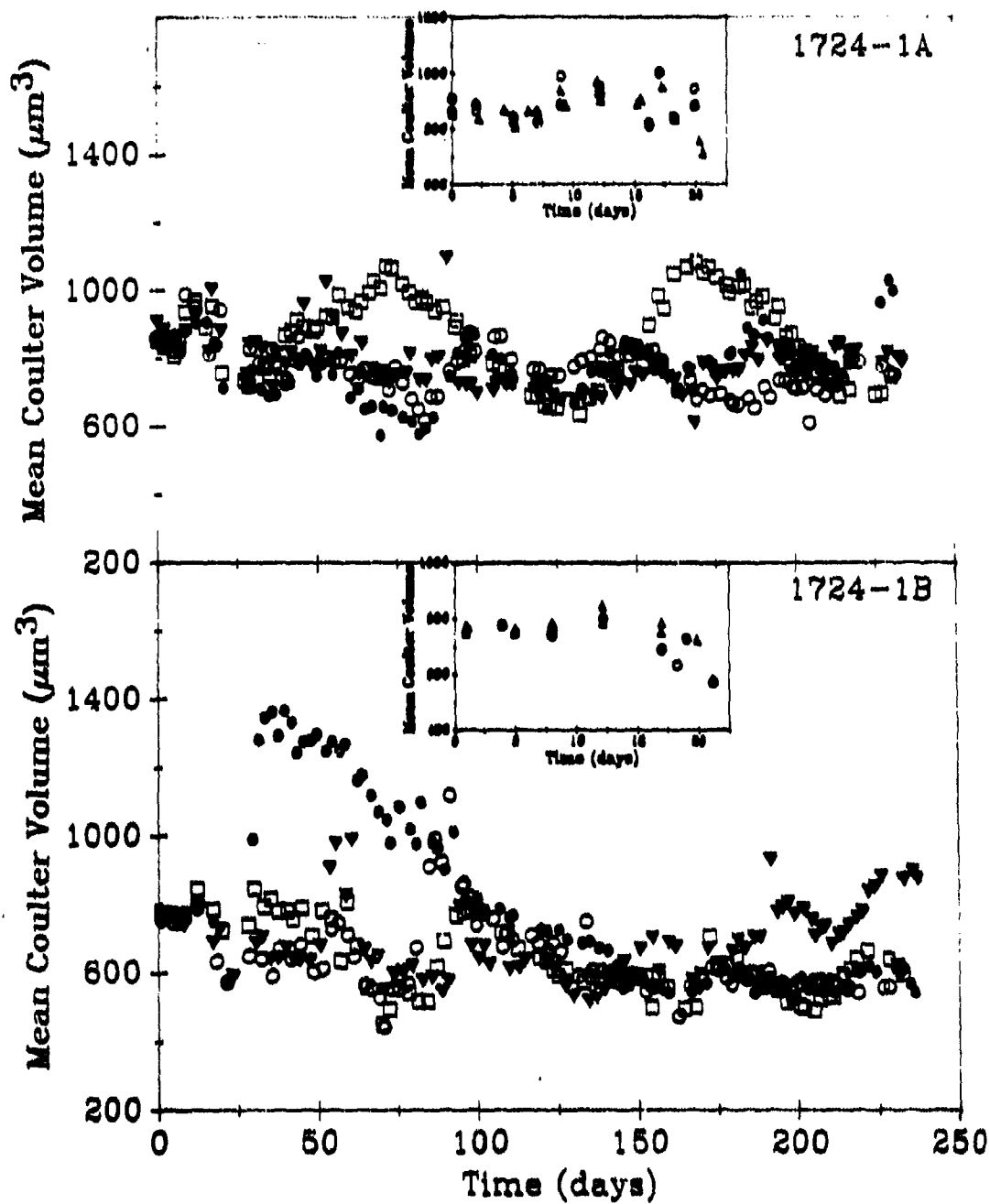
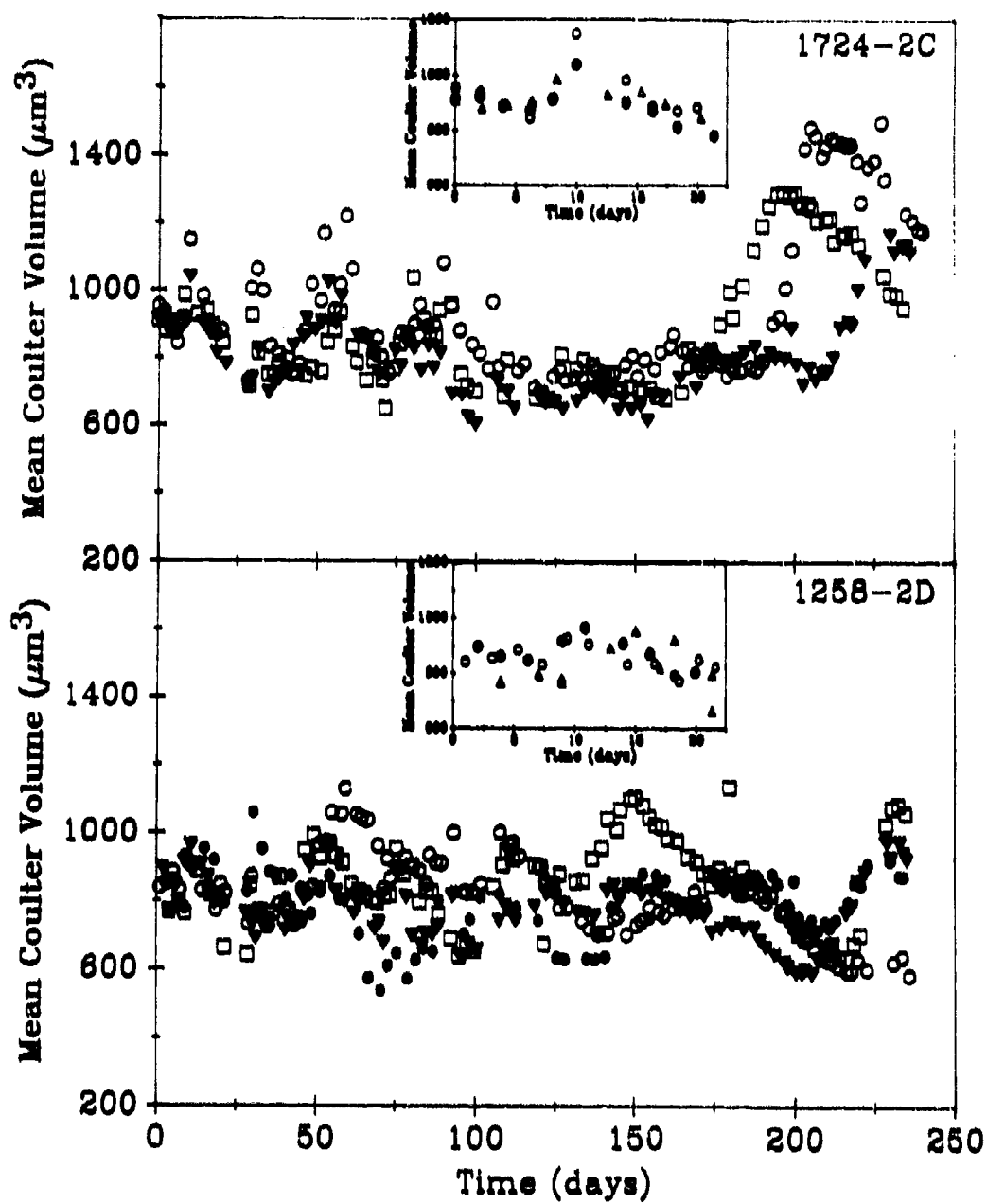


Fig. 11. Mean Coulter volume over time for two sets of populations derived from genetically identical cells of *T. weissflogii* isolate 1724-2 at t=340 days (see Fig. 4A). Each panel represents populations derived from the same grandmother or great-grandmother cell as indicated in Fig. 2. The different symbols represent the three or four cell lines derived from the original genetically identical cells. The insets are a blow up of the mean Coulter volume (note change in scale) during the first 20 days after the initial isolation.





which these isolates were derived (Fig. 4A, beginning at  $t=340$  d). Moreover, the members of each set of replicates initially behaved identically as would be expected of populations composed of genetically identical cells (the slopes and elevations of the replicate cultures were not significantly different for at least twenty days after isolation,  $p>0.05$ , Student's  $t$  test; see insets Figs. 10, 11). Over time, however, the average cell volume in even these cultures diverged as one of the replicates began to increase in mean Coulter volume; this divergence can be seen most dramatically in Figs. 10B and 11A. Note that the maximum size obtained by populations which were initially genetically identical was not constant implying that over time, the genetic composition of these populations had changed.

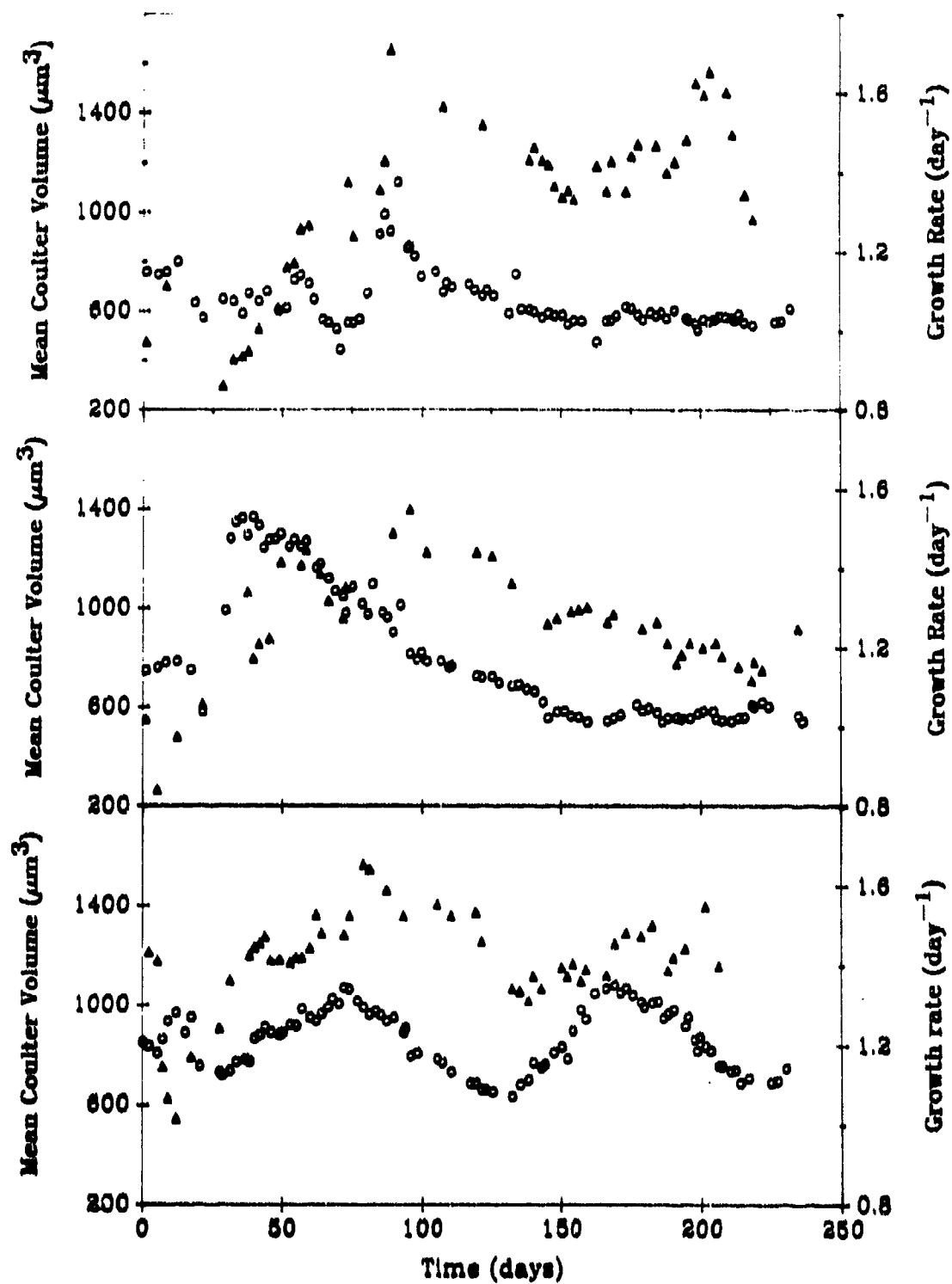
*Population growth rates.* It has repeatedly been shown that post-auxospore cells grow more rapidly than relatively small cells involved in asexual reproduction (e.g. Paasche 1973, Costello and Chisholm 1981). A great deal of scatter was observed in the population doubling times of the *T. weissflogii* isolates derived here and there was no consistent correlation between average cell size and growth rate regardless of the culture examined (Table 1); in only four out of 22 cultures was the relationship significant. When the growth rate of a culture was examined over time rather than simply with respect to absolute cell size, however, a different picture began to emerge. For those cultures in which the mean cell size remained relatively constant, the population growth rates could

vary dramatically with no apparent predictability (Fig. 12A, B). However, during intervals in which discernible oscillations in cell size were apparent, the growth rate was correlated with cell size; newly enlarged cells appeared to grow faster than the smaller cells from which they were created (Fig. 12B, C).

### Discussion

The control of cell size in populations of the centric diatom, *T. weissflogii*, is a quite complicated process. Numerous isolates of this diatom representing a broad range of size distributions and cell lineages, each displayed complex and unpredictable patterns of cell size change during nearly two years of continuous exponential growth. The only feature common to these various size patterns was the rate of decrease in mean cell size, a value which is determined by the physical constraints of the diatom frustule during mitotic divisions and the minimum cell size achieved within a given culture. The rest of the components, i.e., the timing of the onset of sexual reproduction, the rate of increase in average cell size, and the size of the post-auxospores created during the sexual events varied among populations and even within a given population over time in the absence of any obvious selection pressures. We hypothesize that the variability in the patterns of cell size change exhibited by these cultures results from the fact that the genetic composition of the populations varied over time.

Fig. 12. Mean Coulter volume (open circles) and population growth rate (closed triangles) over time in three isolates of *T. weissflogii*. The growth rate data were smoothed over three measurements.



In the absence of any discernible environmental triggers, the transition from asexual reproduction to sexual reproduction is apparently linked with the obtainment of an appropriate cell size. However, this permissive size range can vary among different populations and in some instances, even within a given population over time. Thus the relationship between cell size and the capacity to undergo gametogenesis is not absolute; the ability of two comparably sized cells to exit the mitotic cycle and undergo sexual reproduction appears to be determined by the genotypes of these cells. This result may explain our inability to predict the extent of spermatogenesis induced in these various isolates (Armbrust et al. 1990).

The size of the post-auxospore cells created during the sexual episodes also varied considerably both within and among the different populations regardless of the size of the cells that underwent gametogenesis. The number of generations that elapses between the completion of one sexual event and the initiation of a new one should be determined by the amount of time necessary for these newly created large cells to reach a permissive size range. Thus, the relative values of these two parameters along with the growth rate of the vegetative cells will determine the frequency of sexual events in diatom populations.

The third factor that varied among the different isolates, the rate of increase in mean cell size, is perhaps the most difficult to interpret. At least two possible scenarios exist for what may have

occurred during the intervals of increasing mean Coulter volume. In the first scenario, all the cells within a population are hypothesized to eventually undergo sexual reproduction and auxospore formation during a given interval of time; the rate of increase in average cell size is thus determined by the size of the post-auxospore cells and the rate of entry of vegetative cells into the permissible size range, i.e. the generation time of these asexual cells. In the second scenario, only a portion of the cells are hypothesized to form auxospores but the generation time of these newly enlarged cells is assumed to be much shorter than that of cells which have not yet undergone sexual reproduction. The amount of time for such a cohort of cells with an increased growth rate to dominate a population can be predicted (Wood 1989). Assuming that in the most extreme case only one post-auxospore cell is created in a population of 10,000 cells with an average growth rate of  $1.386 \text{ day}^{-1}$ , the growth rate of the post-auxospore would have to be  $2.372 \text{ day}^{-1}$  or 1.7 times faster than the other cells for its descendants to represent 99 % of the population in 2 weeks and  $1.846 \text{ day}^{-1}$  or 1.3 times faster for its descendants to dominate in 1 month. However, if instead of only one cell, 50 % of this same population formed auxospores, then the growth rate of these cells would only have to be  $1.714 \text{ day}^{-1}$  for this cohort to dominate in two weeks and  $1.539 \text{ day}^{-1}$  or 1.1 times faster than average for the descendants of the post-auxospore cells to dominate the population in one month. Thus, if in fact only a portion of a population undergoes sexual

reproduction, replicate cultures may quickly diverge depending on which cohort of cells comes to dominate which culture.

Given any differences in growth rates in pre- and post-auxospore cells, it becomes impossible to determine based on cell size distributions alone, what portion of a population actually formed auxospores. However, regardless of whether or not the entire population or only a fraction of the population undergoes sexual reproduction, by the time the maximum mean cell size is obtained, all the cells remaining within a culture are the descendants of auxospores (Fig. 9). Thus, the genetic composition of a population before the interval of increasing mean Coulter volume will differ from the composition of the population after the sexual event; consequently either the permissive size range or the new potential size of post-auxospores may have changed. Even more genetic variability can be generated in these populations due to the possibility of spontaneous mutations (Lande 1976). As described previously, any cell with a faster than average growth rate can come to dominate a population over time. If this faster growing cell also possesses a mutation that affects the control of cell size, then this new genotype can be propagated throughout the population during the intervals of asexual reproduction.

This study which began as an attempt to describe the development of cell size over time in diatoms, has uncovered some of the wonderful complexity of these populations. The composition of a population of these organisms even in the absence of any selection

pressures is extremely fluid with the potential for frequent alterations of both the genotypes and phenotypes of its members. Even populations that originate from genetically identical cells can rapidly display dramatically different behaviors. Thus, over the course of a year, the characteristics of a population may undergo numerous transformations, a feature which must be kept in mind as attempts are made to understand the details of the physiology of these organisms.

#### Acknowledgments

Many thanks to Sheila Frankel for her technical assistance, to Dean Jacobsen for his help with the single cell isolations, and to Tina Bartschat for her translations of German articles. The culture work could not have been performed without the help of S. Krolikowski, D. Chen, J. Hwang, T. Helsten, A. Worden and C. Utterback who were supported in part by Sea Grant and the UROP office at MIT.



TABLE 1. Summary of the rates of decrease and increase in mean Coulter volume and the ratio of the maximum volume of a population to the minimum value prior to the initiation of a size increase. All rates reflect the linear regression during an increase or decrease in volume. A generation time of 12 hours was assumed in all calculations<sup>a</sup>.

Clone	Rate of Decrease <sup>b</sup> ( $\mu\text{m}^3 \cdot \text{cell}^{-1} \cdot \text{gen}^{-1}$ )			Rate of Increase <sup>b</sup> ( $\mu\text{m}^3 \cdot \text{cell}^{-1} \cdot \text{gen}^{-1}$ )			Max:Min (range)
	X	± SE	(n) <sup>c</sup>	X	± SE	(n)	
1147-1 <sup>d</sup>	9.8 ± 0.6		(5)	12.1 ± 3.8		(4)	1.6 - 3.5
-2	8.6 ± 0.6		(6)	10.3 ± 1.4		(5)	1.2 - 2.6
920-1	8.2 ± 0.8		(6)	4.5 ± 1.8		(5)	1.2 - 1.8
-2	9.2 ± 0.8		(4)	9.7 ± 3.8		(4)	1.2 - 2.4
444-1	7.2 ± 0.8		(5)	5.5 ± 1.5		(5)	1.2 - 1.9
-2	N. A.						
868-1	4.0 ± 0.8		(3)	2.3 ± 0.2		(4)	1.3 - 1.9
-2	N. A.						
709-1	6.0 ± 2.7		(2)	3.7 ± 1.2		(2)	1.6 - 2.3
-2	5.6 ± 1.6		(2)	5.3 ± 2.0		(2)	2.3 - 2.5
877-1	8.4 ± 1.3		(2)	3.6 ± 0.9		(2)	1.8 - 1.9
-2	N. A.						
877-1	8.4 ± 1.3		(2)	3.6 ± 0.9		(2)	1.8 - 1.9
-2	N. A.						
593-1	7.2 ± 0.7		(2)	9.6 ± 2.8		(3)	1.7 - 2.0
-2	9.6 ± 1.8		(4)	8.6 ± 3.0		(4)	1.3 - 2.1
747-1	8.2 ± 1.1		(2)	10.5 ± 5.1		(2)	1.3 - 2.0
-2	8.0 ± 0.8		(2)	5.2 ± 0.4		(2)	1.5 - 2.2
1724-1	16.4 ± 7.6		(4)	9.0 ± 2.9		(3)	1.8 - 2.1
-2	10.6 ± 0.8		(4)	12.0 ± 3.6		(4)	1.5 - 2.2
834-1	6.6 ± 1.6		(4)	3.8 ± 1.8		(3)	1.4 - 2.5
-2	6.4 ± 1.2		(4)	3.6 ± 0.7		(3)	1.3 - 2.4
637-1	4.8 ± 2.0		(2)	5.5 ± 1.6		(2)	1.6 - 2.2
-2	10.0 ± 2.8		(3)	5.8 ± 1.5		(4)	1.7 - 1.8

Table 1, cont.

- <sup>a</sup> In reality, generation times ranged from approximately 8-19 hours, which does not influence the conclusions.
- <sup>b</sup> Rates of decrease were multiplied by two since upon division, only one of the two daughter cells is smaller than the its mother.
- <sup>c</sup> n refers to the number of increases or decreases in average Coulter volume regressed.
- <sup>d</sup> -1 and -2 refer to the two replicate cultures.
- <sup>e</sup> N. A. or not applicable indicates that at least two increasing or decreasing slopes could not be analyzed.

TABLE 2. Summary of correlation coefficients (r) between population doubling times and mean Coulter volume over time for T. weissflogii isolates maintained exponentially in continuous light and constant temperature. Doubling times were determined from three consecutive fluorescence readings. Only those growth rates which were derived from a regression with  $r^2 > .990$  were considered for the calculation of the correlation coefficients.

Isolate Designation	Replicate 1		Replicate 2	
	r	n	r	n
866	.0268	40	.719*	31
709	.104	37	.0092	42
877	.364	30	.213	33
593	.213	33	.153	37
747	.274	30	.564*	38
1147	.0396	26	.270	36
920	.304	30	.137	34
444	.0564	30	.0263	36
1724	.0149	35	.0449	45
834	.0439	37	.457*	39
637	.599*	37	.1612	37

\*P < 0.05.

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## Appendix

### Effect of Light on the Cell Cycle of a Marine *Synechococcus* Strain





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**Effect of Light on the Cell Cycle of a Marine *Synechococcus* Strain**

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**Light-dependent regulation of cell cycle progression in the marine cyanobacterium *Synechococcus* strain WH-8101 was demonstrated through the use of flow cytometry. Our results show that, similar to eucaryotic cells, marine *Synechococcus* spp. display two gaps in DNA synthesis, at the beginning and at the end of the cell cycle. Progression through each of these gaps requires light, and their durations lengthen under light limitation.**

Predictions of the physiological responses of individual cells to environmental change require an understanding of cell cycle regulation. The environment affects the progression of cells through their various cell cycle stages and therefore influences the duration of the division cycle. Furthermore, many metabolic processes occur during discrete periods of the division cycle, resulting in limited intervals when cells are able to detect and respond to external stimuli. Thus, a complex feedback system exists between cells and their immediate surroundings. We have shown, for example, that the position of light-dependent arrest points in the cell cycles of two unicellular marine phytoplankton, a diatom and a coccolithophorid (36), explains the population growth patterns of these organisms on a range of light/dark cycles (35). Similarly, only with the discovery that photosynthesis and nitrogen fixation are restricted to different portions of the cell cycle (thus, temporally separating nitrogenase activity from oxygen evolution (26)) could the presence of nitrogen fixation in populations of unicellular cyanobacteria be adequately explained (26, 37).

Our interest in the cell cycle of marine *Synechococcus* spp. arose from a desire to understand the observed growth patterns of these cyanobacteria on a diel light/dark cycle. Like many diatoms (7), *Synechococcus* spp. divide predominantly during the light interval (38) rather than the dark, a characteristic which sets these two groups of cells apart from most other phytoplankton. A second unusual characteristic of *Synechococcus* populations is that the frequency of dividing cells becomes constant during the dark interval when cell division is no longer occurring (38), implying that a certain fraction of the population is arrested in the doublet stage during most of the night.

The freshwater cyanobacterium *Anacystis nidulans* has been the focus of numerous cell cycle studies (e.g., 10, 24), and yet, cell cycle regulation in related marine species of unicellular cyanobacteria remains virtually unexplored. Prokaryotic cell cycle models based on *Escherichia coli* predict that DNA will be synthesized continuously throughout the division cycle when the amount of time necessary to complete DNA synthesis and cell division is greater than the generation time of the organism (12, 17, 18, 22). *A. nidulans* appears to contain multiple copies of its genome at growth rates which, according to the *E. coli* model, would not necessitate continuous DNA synthesis (23). The minimum

doubling time observed for marine *Synechococcus* strains can be almost an order of magnitude greater than that of rapidly growing *A. nidulans* or *E. coli* populations. These observations raise the question of whether the cell cycle characteristics of marine *Synechococcus* spp. will be more similar in appearance to the prokaryotic model of *E. coli* or to their rapidly growing prokaryotic relative, *A. nidulans*, or perhaps instead to the relatively slowly growing eucaryotic cells.

The goals of our study were to describe the DNA cycle of *Synechococcus* spp. as a function of the growth rate and to examine light-dependent regulation of the cell cycle in order to predict the behavior of populations of cells in their natural environment. Our emphasis was on the elucidation of this coupling between the natural photocycle and cell cycle progression, rather than on the mechanisms controlling this coupling. The locations of light-dependent processes were identified, through the use of flow cytometry, by examining cell cycle responses to light limitation and by determining the arrest points of populations deprived of light (30, 36). Our findings indicate that, similar to eucaryotic cells, the marine *Synechococcus* sp. displays two gaps in DNA synthesis at each growth rate examined. Moreover, unlike most other unicellular algae, in which only the interval before DNA synthesis is light dependent (27, 30, 33), in the *Synechococcus* sp., cell cycle progression through each of the gaps in DNA synthesis requires light. For simplicity and in accordance with eucaryotic nomenclature, these two gaps will be referred to as G<sub>1</sub> and G<sub>2</sub>, and the interval of DNA synthesis will be referred to as S.

#### MATERIALS AND METHODS

**Culture conditions.** The *Synechococcus* sp. (strain WH-8101), an inshore isolate which lacks phycoerythrin, was obtained from John Waterbury at the Woods Hole Oceanographic Institution and was grown in F2 enriched seawater medium (16). For the light limitation studies, semicontinuous cultures were maintained in exponential growth for a minimum of 17 generations at 25°C under continuous illumination ranging from 22.4 to 174 microeinsteins · m<sup>-2</sup> · s<sup>-1</sup>. A Turner fluorometer was used to monitor daily changes in the in vivo fluorescence of the cultures in order to determine growth rates (5).

For the dark arrest experiment, cultures were grown at 19°C in continuous light at 70 microeinsteins · m<sup>-2</sup> · s<sup>-1</sup> before placement in the dark. During the course of the experiment, changes in the cell number were determined

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with an epifluorescent Zeiss microscope. A sample of culture was diluted with filtered seawater to obtain approximately 25 to 50 cells per field and then filtered at a vacuum pressure of less than 125 mm of mercury onto a Nuclepore filter (pore size, 0.2  $\mu\text{m}$ ). The filter was then sandwiched between a cover slip and a slide with immersion oil and either frozen or counted immediately. An average of 400 cells per filter was counted. In some experiments the cell number was also monitored with an Epics V flow cytometer (488-nm excitation, 680-wide-band emission); a known quantity of standard beads was added to live samples to calculate the amount of sample run through the flow cytometer and thus determine the cell concentration (28).

A series of cultures were also maintained in steady state for 2 weeks on a 14-h:10-h light-dark cycle at a light level of 100  $\mu\text{Einsteins m}^{-2} \text{ s}^{-1}$  and a temperature of 20°C. Average population growth rates were determined by monitoring *in vivo* culture fluorescence at the same time each day to eliminate the effects of diel periodicity on fluorescence yield. During the actual experiment, cell division was monitored by microscope counts as described above.

**Fixing and staining for flow cytometric analysis.** One to ten million cells were harvested by centrifugation at  $11,000 \times g$  for 3 min and subsequent removal of all but 0.1 ml of the supernatant. The suspended pellet was then injected through a 24-gauge hypodermic needle into 10 ml of ice-cold absolute methanol and stored at 4°C for a minimum of 2 h to both fix the cells and remove chlorophyll. Samples prepared in this manner remain stable at 4°C for up to 1 year before analysis (29).

In preparation for the flow cytometric analysis of DNA, 1.5 ml of the methanol-fixed sample was centrifuged at  $11,000 \times g$  for 3 min. The supernatant was removed, and the pellet was washed once with 1.5 ml of 10% phosphate-buffered saline (5 mM  $\text{KH}_2\text{PO}_4$ , 5 mM  $\text{K}_2\text{HPO}_4$ , 0.015 M NaCl; pH 7.4) and resuspended in 1 ml of 10% phosphate-buffered saline. By using a modification of the technique of Crisman and Steinkamp (9), propidium iodide (Sigma Chemical Co.; final concentration, 30  $\mu\text{g} \cdot \text{ml}^{-1}$ ) and RNase A (Sigma; final concentration, 0.66  $\text{mg} \cdot \text{ml}^{-1}$ ) were added to each sample, and the samples were incubated at room temperature for 2 h. After the 2-h incubation, background stain was removed, if necessary, by first filtering the sample through a Nuclepore filter (pore size, 0.2  $\mu\text{m}$ ) and then washing it with 2 ml of 10% phosphate-buffered saline. The washed cells were resuspended in 1 ml of 10% phosphate-buffered saline and analyzed within 4 h with an Epics V flow cytometer.

**Flow cytometric analyses.** Laser excitation at 314 nm was used to measure simultaneously the forward angle light scatter (FLS), which is an indicator of cell size, and propidium iodide fluorescence (emission measured at greater than 590 nm) of individual cells. To increase sensitivity, 1 W of laser power was focused to a spot size of 16.5 by 40  $\mu\text{m}$  with a confocal lens system. A total of 20,000 to 50,000 cells per sample were analyzed in this manner. Uniform fluorescent beads (diameter, 0.9  $\mu\text{m}$ ; Duke Scientific) were added to each sample as an internal standard to monitor drift in the performance of the flow cytometer and to calibrate each fluorescence and FLS measurement so that samples analyzed on different days could be compared.

The data were stored both as single-parameter DNA fluorescence histograms of 256-channel resolution and as two-parameter histograms of correlated FLS and DNA fluorescence of 64  $\times$  64-channel resolution.

**Data analysis.** The data generated with the Epics V flow

cytometer were transferred to an IBM 9000 computer for detailed analysis (D. Vault, Ph.D. thesis, Massachusetts Institute of Technology and the Woods Hole Oceanographic Institution, Cambridge, Mass., 1986). Single-parameter DNA histograms were analyzed by assuming that the two main fluorescence peaks corresponded to  $G_1$  and  $G_2$  cells of either one or two complements of DNA, respectively, and that the region separating these two peaks represented those cells in the process of DNA replication, or the S phase. To calculate the percentage of cells in each of these cell cycle stages, it was assumed that the  $G_1$  and  $G_2$  peaks displayed Gaussian distributions of DNA fluorescence per cell (11) and that the S phase could be approximated as the sum of a series of Gaussian curves (Vault, Ph.D. thesis).

The duration of each cell cycle stage,  $T_{\text{cell}}$ , was calculated from the proportion of the population in each stage,  $P_{\text{cell}}$ , and the doubling time of the population in exponential growth,  $t_d$ , according to the equations of Slater et al. (32):

$$\begin{aligned} T(G_1) &= -t_d / P \ln \{1 - [P(G_1)/2]\} \\ T(G_2) &= t_d / P \ln \{1 + [P(G_2)/2]\} \\ T(S) &= t_d / P \ln \{1 + P(S)[1 + P(G_2)]\} \end{aligned}$$

Two-parameter histograms were analyzed by first counting the cells within a given window. Modes and coefficients of variation (CVs) were then calculated for a single parameter of these selected data. Finally, the mode of the FLS and DNA fluorescence was transformed from a logarithmic scale to a linear scale, calibrated against the 0.9- $\mu\text{m}$  beads, and expressed in relative units.

**Computer model.** The general image of cell cycle regulation of prokaryotic organisms has been extrapolated from the model for *E. coli* proposed by Helmstetter and Cooper (17) and is summarized in Fig. 1. In their scheme the important determinant of cell cycle behavior is the duration of the division cycle relative to the DNA cycle (Fig. 1A and B). We developed a computer simulation based on this *E. coli* model (13, 18) to predict the distribution of DNA per cell for prokaryotic populations displaying a range of growth rates (Fig. 1C and D). The use of this numerical model enabled us to predict how the flow cytometric data would appear if cyanobacteria adhered to the "rules" of *E. coli* and to place boundaries on the relationship between the cell cycle and the DNA cycle in this species.

The input parameters required for each model condition were the population doubling time ( $t_d$ ), the time necessary for a pair of replication forks to travel the entire length of the chromosome (the DNA replication time, or  $t_r$ ), the minimum time elapsed between the completion of chromosome replication and cell division (the septum formation time,  $D$ ), and the CV for the DNA measurement. For slowly growing cells in which the generation time was greater than the amount of time necessary for chromosome replication and septum formation, the duration of either the  $G_1$  or  $G_2$  gap was also needed ( $G_1 + t_r + G_2 = t_d$ ;  $G_2 \geq D$ ).

Three assumptions from the *E. coli* model were central to the formulation of our model. First, it was postulated that each replication fork moved along the chromosome at a constant rate (3, 20). Second, it was assumed that a newly born cell contained a single complement of DNA if the generation time was greater than or equal to the sum of the DNA replication and septum formation times. Third, if the generation time of a cell was less than this sum, then synthesis of a complete chromosome spanned more than a single generation. Newly born cells therefore contained a single complete DNA complement plus one or more partial

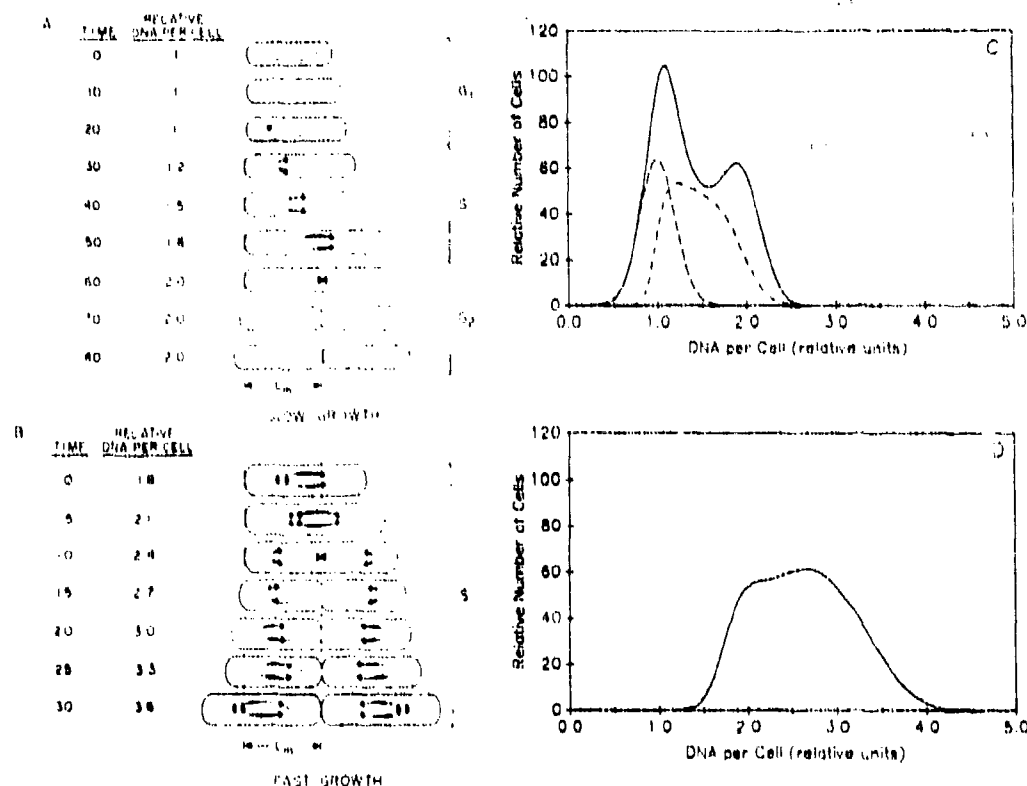


FIG. 1. Prokaryotic model of chromosome replication based on the cell cycle of *E. coli* (adapted from reference 12) (A and B) and the numerical simulations of the distribution of DNA per cell (assuming a CV of  $10^{-1}$ ) in relatively slow (C) and fast (D)-growing populations. In panels A and B,  $L_{min}$  represents the minimum cell length at which septum formation can begin and the triangles represent replication forks on the single circular chromosome. (A) When the generation time of a cell is greater than the amount of time necessary to complete chromosome replication and cell division, gaps in DNA synthesis similar in appearance to the eucaryotic intervals of  $G_1$  and  $G_2$  will occur both before and after the DNA replication period. In this example the durations of  $G_1$  and  $G_2$  are equal and the summation of these intervals is equivalent to the DNA replication time. (B) When the generation time of a cell is less than the amount of time necessary for a replication fork to travel around the chromosome, new rounds of DNA synthesis are initiated before the preceding rounds are completed. In this instance the DNA replication time is 1.3 times as long as the generation time. (C) Simulated distribution of the relative DNA fluorescence per cell of the exponentially growing populations displayed in panel A. The leftmost dashed line represents those cells in  $G_1$ , the dashed line in the center represents those cells in the process of chromosome replication, the dotted line represents cells in  $G_2$ , and the solid line is the sum of the distribution of cells in each of these stages. (D) In the fast-growth case (B), all cells are in the S phase since DNA synthesis occurs throughout the cell cycle. Note that the mean DNA content per cell is higher for this population than that in the slowly growing example.

complements, the synthesis of which began in a previous generation (Fig. 1B).

To calculate the DNA histogram for an asynchronously growing population, the program first subdivided the cell cycle into a number of increments of constant duration and then calculated the DNA amount for each increment. For slowly growing populations ( $t_d \gg t_r + D$ ),  $G_1$  cells contained a single DNA complement,  $G_2$  cells contained two DNA complements, and cells in the process of chromosome replication contained an intermediate amount of DNA linearly related to the amount of time spent in S relative to the total duration of this phase.

To ascertain the relative amount of DNA in a newly born cell ( $DNA_{t=0}$ ) under fast growth conditions, the number of

generations,  $m$ , which have to be spanned in order to complete the synthesis of an entire chromosome was calculated by rounding up to the nearest integer the ratio,  $(t_r + D)/t_d$ . Based on  $m$ ,  $DNA_{t=0}$  was then determined in the following manner. When  $m = 1$ , a newborn cell contains one complete DNA complement,  $A$ . When  $m = 2$ , a newborn cell contains  $A$  amount of DNA in addition to one partial complement,  $B$ , the synthesis of which must be completed  $D$  minutes before division. Since  $B$  must equal 1 at time  $t = t_d - D$ , at time  $t = 0$ ,  $B = 1 - [(t_d - D)/t_r]$  or, upon rearranging,  $B = 1 + [(D - t_d)/t_r]$ . In a similar fashion, when  $m = 3$ , the DNA content in a newborn cell will be  $A + B + 2$  partial complements,  $C$ , each with  $t_d/t_r$  less DNA than  $B$ . At time  $t = 0$ ,  $C = 1 + [(D - t_d)/t_r] - (t_d/t_r)$  or  $C = 1 + [(D - 2 \times$

$t_d/t_r$ . A continuation of this scheme results in the following general equation for determining the amount of DNA within a newborn cell:

$$\text{DNA}_{i+1} = 1 + \sum_{j=1}^m 2^{j-1} [1 + (D - (j-1) \times t_r)/t_r]$$

To calculate the DNA amount for each cell cycle increment in a fast-growth case ( $t_d < t_r + D$ ), the program determined the time at which replication forks began and ended synthesis according to the following equations:

i	start time	finish time	no. of replication fork pairs
1	0	$t_r + D$	1
	0	$t_r$	$2^{i-1}$
m	$(m-1)t_r + t_r + D$	$t_r$	$2^{m-1}$

These equations were derived by assuming that chromosome replication required  $t_r$  minutes and that cell division occurred  $D$  minutes after a complete complement was synthesized. The term  $i$  is an index from 1 to  $m$  designating each of the sets of complements to be synthesized during the cell cycle. The DNA amount for each increment was calculated based on the DNA amount from the previous increment, the increment duration, the DNA replication time, and the number of active replication forks. Once the DNA amount for each cell cycle increment was known, the relative number of cells within each subdivision was determined according to the equations for the age distribution of an asynchronously growing population (8).

The final processing step was to incorporate into the histogram the experimental error associated with the flow cytometric determination of relative DNA fluorescence per cell. The DNA fluorescence for a given DNA amount was assumed to be a Gaussian distribution with a constant CV (11). The subpopulation within each cell cycle increment was thus also distributed into a Gaussian fluorescence curve according to the DNA amount and the CV. The final distribution of the relative cell number versus the relative DNA fluorescence was therefore assembled by summing the fluorescence contributions from each subpopulation.

## RESULTS

**Model predictions.** A range of potential distributions of DNA per cell was examined by employing our computer model to simulate both discrete (Fig. 1A and C) and continuous (Fig. 1B and D) DNA replication within exponentially growing populations. The results of the numerical model indicated that a unimodal distribution of DNA per cell could be obtained under a suite of conditions ranging from continuous DNA synthesis to the presence of either one or two gaps, depending on the CV of the DNA measurements. In other words, little can be said about cell cycle regulation if a unimodal distribution is obtained. However, a bimodal distribution of DNA per cell (Fig. 1C) could be obtained only under two circumstances: either two gaps,  $G_1$  and  $G_2$ , existed during the cell cycle or only a single gap,  $G_2$ , was present after completion of DNA synthesis. It was thus necessary to develop criteria to determine whether a  $G_1$  peak in addition to the  $G_2$  peak was present. Our first step was to simulate the DNA distribution of an unsynchronous population with a constant DNA synthesis rate, no gaps, and a CV of 0. Since the S phase occupies the entire cell cycle, the

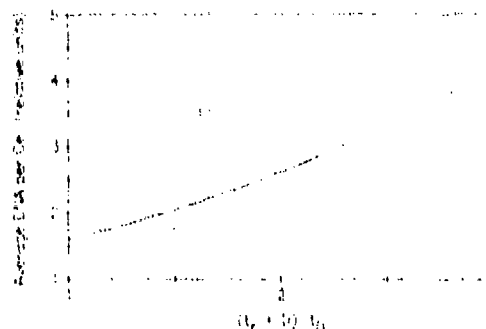


FIG. 2. Plot of population average DNA per cell in asynchronous populations displaying continuous DNA synthesis. The results were obtained by averaging the DNA histograms determined using our numerical model.  $t_r$  is the amount of time necessary for a replication fork to travel completely around the chromosome,  $D$  is the septum formation time, and  $t_d$  is the generation time of an individual cell. In this example the assumptions of the *E. coli* model were observed such that  $t_r$  was 40 min and  $D$  was held constant at 20 min. These results are in agreement with analytical solutions such as those presented by Helmstetter et al. (10) and Skarstad et al. (31a).

ratio of the number of cells entering S to those leaving S is 2. The addition of a  $G_1$  peak to this population decreases this ratio to below 2. Since a CV of 0 is unrealistic for flow cytometric measurements of DNA content, we next simulated the DNA distribution of a population with a constant CV of greater than 0 and only the S and  $G_2$  stages. Under these circumstances a measure of the number of cells entering S relative to those leaving S can be obtained by determining the ratio of the maximum peak height to the saddle height between the two peaks. Using our numerical model, we then simulated DNA distributions with a range of CVs and  $G_2$  proportions and found that the maximum ratio of the peak height to the saddle height is 2. The  $G_1$  distribution is a Gaussian curve, with a peak at a DNA amount less than or equal to the peak of the S distribution (Fig. 1C). The addition of a  $G_1$  peak will therefore increase the peak-to-saddle ratio. Thus, if the observed ratio for a population is greater than 2, this bimodal distribution must include a  $G_1$  peak. Populations with a  $G_1$  phase may have a ratio less than 2 if the error in DNA measurement is high, but populations with a ratio greater than 2 must contain cells with a  $G_1$  phase.

The presence of gaps in DNA synthesis implies that the duration of the DNA replication cycle is less than that of the division cycle. Thus, a newly born cell would contain a single complement of DNA and no cell would ever possess more than two complements of the genome (Fig. 1A and C). On the other hand, if the DNA replication time is longer than the division cycle, a newborn cell would inherit replication forks from the mother cell and would therefore always contain more than a single complement of DNA (Fig. 1B and D). Moreover, if DNA is synthesized continuously, a positive correlation would exist between the growth rate and the DNA content per newborn cell such that as the growth rate increases, the average DNA content per cell would also increase (Fig. 2).

**Light-limited growth.** To determine whether DNA was synthesized continuously throughout the division cycle and how cell cycle progression was modified as a function of the

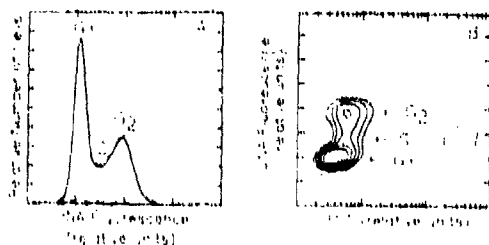


FIG. 3. Representative DNA distributions of *Synechococcus* strain WH 8101 cells stained with the DNA fluorochrome propidium iodide. (A) Single-parameter histogram of the relative number of cells versus the relative DNA fluorescence per cell. The dotted line represents the experimental data and the solid line represents fitted data (Vaulot, Ph.D. thesis). (B) Two-parameter histogram of relative DNA fluorescence per cell versus FLS (size) for the same sample as in panel A. The contour levels relative to the total number of cells analyzed are 0.079, 0.126, 0.202, 0.323, and 0.517.

growth rate, light-limited *Synechococcus* populations were maintained in steady-state growth with doubling times ranging from 11 to 55 h and then analyzed by flow cytometry. Regardless of the growth rate examined, a bimodal distribution of DNA fluorescence per cell was obtained and the ratio of the left peak height to the height of the saddle between the peaks was always greater than 2 (Fig. 3). These two factors indicate that the cell cycle of each of these *Synechococcus* populations contains a discrete interval of DNA replication bounded on either side by a gap in synthesis. This bimodal distribution, reflecting cells with one and two complements of DNA, is apparent in both the single-parameter DNA histograms (Fig. 3A) and the two-parameter histograms of FLS and relative DNA fluorescence (Fig. 3B). Cells in the process of chromosome replication display an intermediate amount of DNA fluorescence, again apparent in both types of histograms (Fig. 3). A critical cell size requirement for the entry of the  $G_1$  cells into S is suggested by the increased average FLS of the S cells relative to the  $G_1$  cells (Fig. 3B). The mode DNA fluorescence of both the  $G_1$  and  $G_2$  peaks did not differ significantly at each of the various growth rates (Table 1), further suggesting that the DNA copy number does not increase above  $2n$  over this range of growth rates (Fig. 1A and B).

The DNA histograms were further analyzed to determine the steady-state proportion of cells in each cell cycle stage at

TABLE 1. Relative DNA fluorescence of  $G_1$  and  $G_2$  peaks in light-limited cultures at a range of growth rates

Doubling time (h)	$G_1$ mode fluorescence <sup>a</sup> $\pm$ SD	$G_2$ mode fluorescence <sup>a</sup> $\pm$ SD
14.4	66 $\pm$ 4 <sup>b</sup>	119 $\pm$ 6 <sup>c</sup>
23.8	68 $\pm$ 1	121 $\pm$ 6
33.4	71 $\pm$ 4	128 $\pm$ 7
55.2	66 $\pm$ 3	119 $\pm$ 8

<sup>a</sup> The mode relative fluorescence refers to the channel number of the 256-channel-resolution DNA fluorescence histogram.

<sup>b</sup> Note that in each instance the  $G_1$  mode fluorescence is slightly less than twice that of the  $G_2$  mode. This has been noted for many cell types and is attributed to the specifics of the flow cytometric system used (15).

<sup>c</sup> The single factor analysis of variance test revealed that the means are not significantly different ( $F_{0.05, 3, 4} = 1.0$ ;  $P > 0.25$ ).

<sup>d</sup> The single factor analysis of variance test revealed that the means are not significantly different ( $F_{0.05, 3, 4} = 0.056$ ;  $P > 0.25$ ).

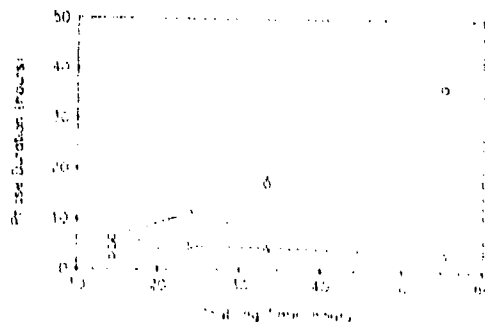


FIG. 4. Duration of each of the cell cycle stages  $G_1$ , S, and  $G_2$  versus the doubling time of light-limited populations of *Synechococcus* strain WH 8101. The duration of each of the stages was calculated as described in Materials and Methods. Symbols:  $\circ$ , S cells;  $\Delta$ ,  $G_1$  cells;  $\square$ ,  $G_2$  cells. The error bar which is larger than the size of the symbol represents the standard deviation for replicate samples.

the various growth rates ( $\mu = 1.5$  to  $0.30$  day<sup>-1</sup>) in order to calculate the duration of each cell cycle stage (see Materials and Methods). We found that the length of the S interval remained constant regardless of the total length of the cell cycle (Fig. 4). The intervals before and after DNA synthesis expanded as growth became more light limited, implying that progression through both of these stages is light dependent.  $G_1$  expanded to a greater extent than  $G_2$ , increasing sixfold as the doubling time increased from 14 to 55 h (Fig. 4).

**Dark arrest and release.** The results of the light limitation experiment imply that cell cycle progression through both  $G_1$  and  $G_2$  requires light whereas the S interval transit is light independent. This suggests that cells placed in the dark should arrest in their cell cycle during  $G_1$  or  $G_2$ , but not S. To test this possibility, cells growing exponentially in continuous light with a doubling time of 11 h were placed in darkness until cell cycling ceased and then returned to continuous light conditions. DNA fluorescence and FLS were examined during both the light and the dark intervals to determine cell cycle dynamics. Cell division appeared to cease immediately upon placement of the population into the dark (Fig. 5A). During the first few hours in the dark, the mode FLS signal of the dark-arrested cells decreased from 0.23 relative FLS units to a minimum of 0.13 (Fig. 5B). This decreased FLS was then maintained throughout the remaining 70 h of darkness. When the population was reexposed to light, the FLS of the cells eventually returned to the pre-dark value of 0.23 relative FLS units.

As predicted from the results of the light limitation experiment, once cell division ceased in the dark (Fig. 5A), cells were blocked in both  $G_1$  and  $G_2$  (Fig. 5C). For reasons that are not yet clear, the CV of DNA fluorescence of the  $G_1$  peak increased in these dark-blocked cells, resulting in an overestimate of the proportion of S-phase cells during the dark interval. We were thus unable to follow the cells in S once division ceased in the dark and could not determine unambiguously whether cells were blocked in synthesis in the dark. However, the fact that the percentage of cells decreased in  $G_1$  and increased in  $G_2$  during the dark when cell division was no longer occurring suggests that cells can progress through S in the dark.

A prolonged dark arrest as in the experiment described above is often used to synchronize populations of cyanobac-

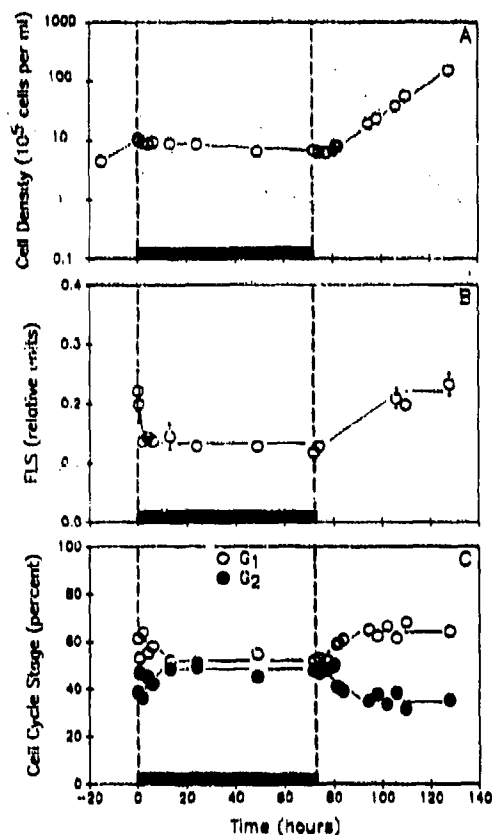


FIG. 5. A culture of *Synechococcus* strain WH-8101 growing exponentially in continuous light with a population doubling time of 11 h was placed in the dark for 72 h and then released into continuous light. The darkened bar indicates the duration of the dark interval. The cell number (A), FLS (B), and the percentage of cells in  $G_1$  and  $G_2$  (C) were measured with a flow cytometer during both the dark and the light intervals. Due to the increased CV of the  $G_1$  cells in the dark, these DNA distributions were assumed to represent only two cell cycle stages ( $G_1$  and  $G_2$ ). Symbols in panel C:  $\circ$ ,  $G_1$  cells;  $\bullet$ ,  $G_2$  cells. In panel B the error bars larger than the symbol size represent the standard deviation for replicate samples.

teria (19, 26). When our dark-arrested *Synechococcus* cells were released into the light, however, there was no evidence of synchronous growth. An initial lag was apparent, but once the cells initiated division, exponential growth resumed rapidly (Fig. 5A). Flow cytometry results also confirmed the absence of synchronized growth in the population after reexposure to continuous light; at all times after release of the cells into the light, both  $G_1$  and  $G_2$  cells were present (Fig. 5C). A period never existed when the culture was dominated by a single phase, as would be expected in a synchronized culture.

**Diel light/dark cycle entrainment.** An alternative means of synchronizing phytoplankton populations is to maintain cultures on a repeating light/dark cycle (1, 34). Obviously,

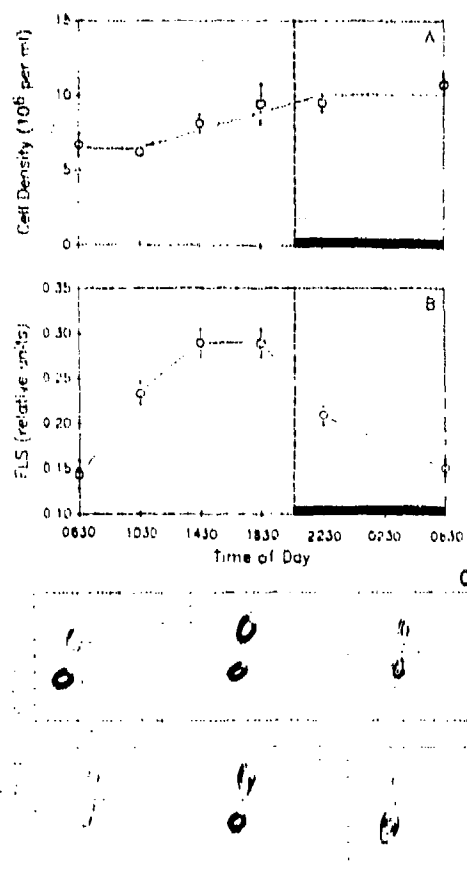


FIG. 6. Time course of the cell division (A), FLS (B), and DNA distributions (C) of *Synechococcus* strain WH-8101 over 24 h of a 14-h:10-h light/dark cycle. The darkened bar represents the dark interval. In panels A and B the error bars larger than the symbol size represent the standard deviation for replicate samples. In panel C the contour levels relative to the total number of cells analyzed are 0.125, 0.175, 0.225, 0.30, 0.375, and 0.4375.

entrainment of a population to a diel light/dark cycle is very different from an interruption of exponential growth in continuous light with darkness; moreover, a light/dark cycle is the "real world" experience of cells. To expand upon the results of the previous experiments, cell division, FLS, and DNA distributions were measured over 24 h in a *Synechococcus* population entrained to a 14-h:10-h light/dark cycle. The cell division pattern for strain WH-8101 observed over this single cycle was similar to that described by Waterbury et al. (38) for a number of cycles in which *Synechococcus* strain WH-7803 divided predominantly during the light interval (Fig. 6A). Over the course of the dark interval, the FLS of the cells declined to its minimum value (Fig. 6B). Upon reexposure to the light, cell division did not begin again for

an additional 4 h (Fig. 6A), while during this same 4-h interval the mode FLS of the cells began increasing to its peak obtained after about 8 h of light (Fig. 6B). This same general trend in FLS variation over the diel cycle has been observed in *Synechococcus* field samples (unpublished data).

An analysis of the cell division pattern alone (Fig. 6A) might lead one to hypothesize that a light/dark cycle can synchronize a *Synechococcus* population. However, both  $G_1$  and  $G_2$  cells were present at all times throughout the diel cycle, including the 10-h interval in the dark. This is consistent with the results obtained in the dark arrest experiment and negates the possibility of a truly synchronized culture. These results also substantiate the hypothesis that cell cycle progress through both  $G_1$  and  $G_2$  is light dependent. Again, the exact percentage of the population in the process of DNA synthesis during the night is probably overestimated due to the increased CV of the  $G_1$  peak. However, it was still apparent from the two-parameter histograms (Fig. 6C) that the number of cells involved in chromosome replication, as indicated by those cells with an intermediate amount of DNA fluorescence, was at a minimum during most of the night. This was followed by a peak in DNA synthesis when those cells blocked in  $G_1$  in the dark were released into the light. The  $G_2$  cells were the first to divide upon release into the light; however, the lights went off before the majority of these cells could also initiate DNA synthesis.

#### DISCUSSION

Our increased understanding of general cell cycle characteristics of *Synechococcus* sp. aids in interpreting many distinguishing features of population growth in this organism. At doubling times ranging from 11 to 55 h, *Synechococcus* strain WH-8101 displays a discrete interval of DNA synthesis, and cell cycle progression both before and after this interval requires the action of biosynthetic processes dependent upon light energy. Thus, when cells are grown on a light/dark cycle, the repeating intervals of darkness force a partial phasing of the DNA and division cycles. DNA synthesis occurs primarily during a limited period in the light when those cells which were blocked in  $G_1$  in the dark are able to initiate chromosome replication, and cell division occurs mainly during the light interval when the arrested  $G_2$  cells are able to divide. This light dependency of progress through the  $G_2$  stage could explain observations of Waterbury et al. (38). They note that in marine *Synechococcus* strain WH-7803, the proportion of dividing cells (which would include our  $G_2$  population) remains at a constant value during the night when the cells are no longer dividing. This suggests that a portion of the population is blocked in the doublet stage during most of the night, and thus the frequency-of-dividing-cells method cannot be used with these organisms to calculate growth rates in the field (6, 25).

The fact that *Synechococcus* sp. arrests in both  $G_1$  and  $G_2$  when placed in the dark implies that the population will divide asynchronously upon reexposure to light. Our results indicate that *Synechococcus* sp. behaves exactly as predicted, after an initial lag upon release into the light, cell division quickly becomes exponential. In contrast to our results, marine cyanobacteria *Synechococcus* strains BG 43511 and 43522 can be synchronized by placing an asynchronous population in the dark for an extended period (26). In addition, both Herdman et al. (19) and Asato (2) have shown that *A. nidulans* displays a comparable degree of synchronous growth upon release into the light after dark exposure. However, Asato (2) and Herdman et al. (19) also

describe data which indicate that both  $G_1$  and  $G_2$  cells are present within *A. nidulans* populations during the dark arrest; yet somehow, synchronous division is still achieved after the dark block is released. To explain their results, these authors postulated that progress through each cell cycle stage in the light is differentially affected by a previous exposure to darkness (19, 24). A nonuniform effect of darkness on transit through the different cell cycle stages has also been observed in diatoms and was attributed to an interruption of the silicon transport system by darkness (36).

Cell cycle progression through  $G_2$  is unconditional in most cell types (21), except for the marine diatoms which are known to have both light- and silica-dependent block points during this interval (30, 36). The fact that two such fundamentally different organisms as the diatoms and *Synechococcus* spp. both divide in the light and possess a light requirement at the end of their cell cycle substantiates the hypothesis that a causal relationship exists between the location of light-dependent processes and overall population growth patterns (36).

The actual specifics of the regulation of the cell cycle in *Synechococcus* spp. are more complicated than has been presented thus far. Theories of cell cycle regulation in prokaryotes rely heavily on the *E. coli* model put forth by Helmstetter and Cooper (17). Cell cycle studies conducted to date with cyanobacteria indicate that apparently these prokaryotes do not always adhere to the rules of the *E. coli* model. For example, Mann and Carr (23) found that the DNA content of *A. nidulans* increased up to 16-fold with an increasing growth rate even though the growth rates examined reflected generation times greater than the chromosome replication interval. Under these circumstances, continuous DNA synthesis and multiple replication forks would not be predicted (12, 17). In the study of Mann and Carr (23), the generation time of *A. nidulans* was approximately 2.3 h, which is about twice its chromosome replication period (19). Similarly, our 11-h doubling time of strain WH-8101 is approximately twice its estimated chromosome replication interval of 5 h (Fig. 4). Yet regardless of the comparable relative growth rates, the DNA content of *A. nidulans* increased exponentially with an increasing growth rate, while that of WH-8101 did not. An increased genomic content at relatively slow growth rates has also been reported for the marine cyanobacterium *Agmenellum quadruplicatum*, which can possess up to three copies of its chromosome at generation times of either 7 or 20 h (31), both of which are presumably greater than the synthesis interval in this organism.

An additional conflict between cyanobacteria and the *E. coli* model is that *A. nidulans* appears to possess simultaneously both a discrete interval of DNA synthesis (2, 19) and multiple copies of its genome (23). It is difficult to reconcile the existence of multiple copies of the genome and  $G_1$  and  $G_2$  arrest points at generation times greater than the chromosome replication interval. Apparently, the presence of gaps in DNA synthesis and multiple chromosome copies are not mutually exclusive.

If the *E. coli* model is not a suitable paradigm for the cell cycle behavior of cyanobacteria, perhaps chloroplasts, which have been hypothesized to have a cyanobacterial origin (14), could serve as a more appropriate model. Chloroplasts are known to contain multiple copies of their genome, the number of which varies as the chloroplast develops (4). Little is known, however, about the regulation of the DNA and division cycles within these organelles as well. An enhanced understanding of cell cycle regulation in cyanobacteria could be gained by determining analytically



the relationship between the growth rate and the absolute number of chromosomes per cell. It would then be possible to determine whether marine *Synechococcus* spp. can ever possess both a discrete interval of DNA synthesis and multiple copies of the genome (13, 31, 39).

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